ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 2, pp. 188–193. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © G.G. Anan'ko, T.V. Teplyakova, 2011, published in Mikrobiologiya, 2011, Vol. 80, No. 2, pp. 200–206.

EXPERIMENTAL ARTICLES

Factors Responsible for Transition of the *Duddingtonia flagrans* Carnivorous Fungus from the Saprotrophic to the Zootrophic Nutrition Type

G. G. Anan'ko[1] and T. V. Teplyakova

State Research Center of Virology and Biotechnology VECTOR, Ministry of Public Health and Social Development of Russian Federation, Koltsovo, Novosibirsk oblast, Russia

Received June 2, 2010

Abstract—Quantitative investigation of the factors responsible for trap formation in the nematophagous fungus *Duddingtonia flagrans* F-882 in submerged liquid culture was carried out. The data obtained suggest a complex program for the regulation of zootrophic nutrition in *D. flagrans*. Optimal concentrations of such carbon and nitrogen sources as sucrose (0.4%), ammonium ions (0.2%), and tryptone (0.2%) promote trap formation in the case of contact with the nematodes *Panagrellus redivivus*. Increased concentrations of these compounds, however, inhibit trap formation. The sensitivity of the mycelium to nematode excreta depends on the state of the culture and is increased under limitation by certain nutrient components or in the course of prolonged starvation. A direct correlation was found between the number of caught nematodes and the number of chlamydospores formed on the mycelium. The nutrients obtained from the nematode biomass are used for formation of additional chlamydospores (on average, about 20 chlamydospores per nematode). Environmental and evolutionary aspects of the role of zootrophic nutrition in carnivorous fungi are discussed.

Key words: Duddingtonia flagrans, traps, chlamydospores, nematophagous fungi, zootrophic nutrition.

DOI: 10.1134/S0026261711020020

Carnivorous (nematophagous) fungi, apart from saprotrophic growth on plant and animal debris, are able to catch and kill soil nematodes to feed on their content. Thus, in the structure of soil microcenoses, carnivorous fungi play the roles of both predators and decomposers. The fungi may act in either of these roles, depending on environmental conditions. Although the manner and mechanisms of nutrition in carnivorous fungi have been investigated, some specifics of these processes remain unclear [1]. Researching the relationships between carnivorous fungi and their prey improves our understanding of the structure of soil microcenoses, including the role of nematophagous fungi. Knowledge of the factors controlling their life cycle will make it possible to establish the evolutionary role of zootrophic nutrition in fungi.

Moreover, such research is of considerable applied importance. Carnivorous fungi have long been used against phytoparasitic nematodes [1]. Research on the application of carnivorous fungi, mostly *Duddingtonia flagrans*, against animal helmintoses and for improving the infective background of pastures has recently been intense [2]. Attempts at practical application of carnivorous fungi against parasitic nematodes have sometimes been unsuccessful. The failures resulted mainly from poor understanding of the physiology and ecology of the fungi used as a base for biopreparations. Understanding of the factors controlling various stages of the fungal life cycle is required for successful application of nematophagous fungi in agriculture.

In surface cultures, *D. flagrans* forms an aerial mycelium, which develops thin-walled two-cell conidia after 1–2 days and chlamydospores with thick rigid walls after 3 days. While conidia lose their viability after several months, chlamydospores remain viable for several years. Adhesive rings for capturing the nematodes are formed by both aerial and submerged mycelium on contact with the nematodes or their excretions.

The goal of the present work was investigation of the major factors responsible for transition from the saprotrophic to the zootrophic type of nutrition in *D. flagrans*.

MATERIALS AND METHODS

Subject of research was *Duddingtonia flagrans* strain F-882 deposited in the collection of the Vector State Research Center of Virology and Biotechnology. The strain was maintained by transfers on peptone-maize agar (PMA). The fungus was grown in liquid culture in 750-ml flasks on a rotary shaker (180 rpm) at 28°C. The MK medium contained the following: molasses, 30 ml; maize extract, 7.5 ml; KH_2PO_4, 5 g;

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

NH_4NO_3, 5 g; $MgSO_4$, 2 g; and tap water, to 1 l; pH 6.8 [1]. Liquid cultures were inoculated with the spores obtained from the washout of the PMA-grown surface culture.

The **test object** was the nematode *Panagrellus* redivivus.

The samples for trap-developing experiments were obtained in 200-ml conical flasks with 25 ml of the medium grown for 2 days on a rotary shaker (180 rpm) at 28°C. Each variant was carried out in three iterations. The traps were enumerated under a 400× Jenaval microscope, and the average results for 40 fields of view were determined.

The *conditioning medium* was prepared as follows: the nematodes *P. redivivus* were stored in water for 4 days (1000 organisms/ml) and then removed by filtration through a paper filter. The fungal culture samples were supplemented with the required amount of the conditioning medium and antibiotics (ampicillin, 0.5 mg/ml, and benzylpenicillin, 1.0 mg/ml). The composition of the conditioning medium was not investigated.

Chlamydospore content in the sample was determined after 30-s sonication (20 μm) by counting in a Goryaev chamber.

RESULTS AND DISCUSSION

Most works on carnivorous fungi have been carried out with surface cultures grown on agarized media [3, 4]. They, however, exhibit extreme heterogeneity, since different sites of the fungal culture differ in age and physiological state. The composition of nutrients and biologically active compounds differs in different sites within an agarized medium. The results of quantitative investigation on the physiology of carnivorous fungi are therefore often imprecise and vary from author to author.

Mixed liquid culture is a good alternative to surface cultures, since it ensures uniform distribution of the nutrients and their availability to the mycelium. In our experiments, *D. flagrans* was grown in flasks on a temperature-controlled rotary shaker. Trap formation by the nematophagous fungus *Arthrobotrys oligospora* in liquid medium was investigated by a similar technique [5]. Stirring results in creation of homogeneous conditions and therefore in the uniformity of the culture. In MK medium, *D. flagrans* developed a loose mycelium with a high biomass density (up to 12 g of dry biomass per liter). We focused on the conditions for transition of the fungus to zootrophic nutrition, i.e., the conditions for formation of the nematode traps. *D. flagrans* F-882 forms traps both in submerged and surface cultures, making it possible to carry out quantitative investigation of the parameters controlling this process.

Critical concentration of the nutrient medium for trap formation. We determined that *D. flagrans* F-882 did not form traps spontaneously. Trap formation was induced only by contact with nematode excreta. This type of induction is known for many carnivorous fungi [6]. However, the presence of nematodes or their excreta was not the only factor controlling trap formation. Introduction of the nematodes to MK-grown liquid culture of *D. flagrans* did not result in formation of traps. Rich media are known to inhibit trap formation in a number of other fungi [7].

We have shown that, in the culture of *D. flagrans* F-882 grown in MK medium and then diluted with water, contact with the nematodes induced trap formation. In a series of experiments, the inoculum (3- to 4-day liquid culture, 20% of the final volume of the liquid) was either diluted with water and fresh MK medium at different ratios or supplemented with car-

Table 1. Effect of different concentrations of carbon and nitrogen sources on trap formation by *D. flagrans*

no.	Medium*	Number of traps**
1	80% water, no fresh medium	++
2	70% water, 10% fresh medium	++
3	60% water, 20% fresh medium	++++
4	40% water, 40% fresh medium	++
5	35% water, 45% fresh medium	–
6	80% fresh medium	–
7	0.2% sucrose	++
8	0.4% sucrose	++++
9	0.8% sucrose	+++
10	1.2% sucrose	+
11	1.6% sucrose	–
12	0.2% NH_4NO_3	++++
13	0.4% NH_4NO_3	+++
14	0.8% NH_4NO_3	+
15	0.2% $NaNO_3$	+
16	0.4% $NaNO_3$	+
17	0.8% $NaNO_3$	+
18	0.2% NH_4Cl	++++
19	0.4% NH_4Cl	++++
20	0.8% NH_4Cl	+++
21	0.2% tryptone	++++
22	0.4% tryptone	++
23	0.8% tryptone	+
24	1.0% tryptone	–

Notes: * All samples were supplemented with the nematodes *P. redivivus*, 100 individuals/ml.

** No traps (–), sporadic traps (+), two to five traps per field of view (++), five to ten traps per field (+++), over ten traps per field (++++).

Table 2. Sensitivity of *D. flagrans* cultures with different histories to nematode excreta

no.	Medium	Number of traps*				
		5% CM	10% CM	20% CM	40% CM	80% CM
1	Balanced medium	–	–	–	++	++++
2	Sucrose medium	–	–	++	++++	++++
3	Old culture	+	+++	++++	++++	++++

* No traps (–), sporadic traps (+), up to 5 traps per field of view (++), 5–10 traps per field (+++), over 10 traps per field (++++). CM stands for conditioned medium.

bon and nitrogen sources. The nematodes *P. redivivus* were added to all samples (Table 1).

The number of traps in submerged culture peaked after two days at 28°C; i.e., it occurred at about the same rate as in the surface culture [3]. The critical nutrient concentration was within the 40–45% range of their concentration in full-strength medium (Table 1). Addition of small amounts of fresh medium to the inoculum induced trap formation, while high concentrations of the medium (~45%) completely inhibited their formation. Under such conditions (samples nos. 5 and 6), the nematodes remained viable for a long time, while in samples nos. 1–3 they all were dead after 2–3 days.

These results provided no information concerning the nature of the compounds inhibiting trap formation. The effect of the individual components of the medium on trap formation was therefore investigated.

Critical concentrations of different carbon and nitrogen sources. Molasses—a mixture of different carbohydrates, mostly sucrose (up to 50%)—is the carbon source in MK medium. Maize extract and ammonium nitrate are the nitrogen sources. Molasses and maize extract have a complex composition. Molasses contains also nitrogen compounds (amino acids), while maize extract contains significant amounts of lactic acid [8]. In order to determine the role of carbohydrates and nitrogen compounds in trap development, the effect of sucrose, ammonium, and nitrate ions was studied. Tryptone, a nitrogen source of animal origin, with elevated levels of amine nitrogen (amino acids and peptides) was also investigated.

Table 1 shows that moderate concentrations of all these compounds (except for sodium nitrate) promoted trap formation. However, high concentrations of the same compounds inhibited development of the traps. The optimal concentrations resulting in the maximal number of traps formed were 0.4, 0.2, and 0.2% for sucrose, ammonium chloride, and tryptone, respectively. Ammonium ions (0.2–0.8%) increased the number of traps. Nitrate ions, however, exhibited a certain inhibitory effect. In the case of ammonium nitrate (0.2–0.4%), the incentive effect prevailed.

While the critical addition of fresh medium (45%) equivalent to approximately 0.7% sucrose inhibits trap formation completely, the same amount of sucrose does not inhibit trap formation. The sugars of molasses and nitrogen present in the complete medium probably act as synergists.

The reaction to tryptone shows that *D. flagrans* mycelium is especially sensitive to amino acids, which stimulate trap formation at low concentrations and inhibit it partially or completely at higher concentrations. *Arthrobotrys oligospora* is also known to form no traps on media with three amino acids (histidine, methionine, and tryptophan) and some vitamins [9].

Thus, two requirements are necessary for initiation of trap formation. First, the concentrations of carbon and nitrogen sources should be below a certain level. Second, the mycelium should be in contact with the nematode excreta. These findings suggest technological recommendations for the application of *D. flagrans*-based biopreparations. While excessive introduction of organic and mineral nitrogen fertilizers may inhibit trap formation, thus decreasing the efficiency of the preparation, moderate introduction of ammonium-containing fertilizers will stimulate trap development. This will lead to lower consumption of the preparation at the same level of its efficiency.

Effect of the history of the culture on mycelial sensitivity to nematode excreta. The data on the sensitivity of the mycelium to nematode excreta are of interest. Three samples of *D. flagrans* mycelium were studied, namely, fresh 4-day culture grown in the balanced MK medium, fresh 4-day culture grown in 3% sucrose solution (nitrogen limitation), and MK-grown culture after incubation for 3 months at 4°C. Each sample was supplemented with 20% of the relevant culture and an aliquot of conditioning medium containing nematode excreta and water (Table 2).

The cultures exhibited different responses to nematode excreta. The old culture (no. 3) with the highest degree of limitation was the most sensitive. The culture incubated in pure sucrose-containing medium (under nitrogen limitation) also exhibited relatively high reactivity. The fresh culture grown in rich balanced medium was the least sensitive to nematode excreta. Thus, the physiological state of the culture has a significant effect on its reaction to excreta of the nematodes. Limitation by one or several nutrient components makes the mycelium sensitive to the excreta.

Table 3. Chlamydospore yield depending on the amount of nematodes added to *D. flagrans* culture grown in an unbalanced medium

no.	Nematodes added, individuals/ml	Chlamydospores, 10^4/ml	Chlamydospore yield relative to the control	Chlamydospore quality
1	Control, no nematodes	10.6 ± 1.5	1	Immature, with thin walls
2	400	20.7 ± 2.6	2	Mature, with thick dark walls
3	800	20.9 ± 3.1	2	Mature, with thick dark walls
4	1600	21.4 ± 2.8	2	Mature, with thick dark walls

Our results suggest a complex program for regulation of zootrophic nutrition in *D. flagrans*. Traps are not formed when the nematodes are scarce or absent in the medium. Prolonged starvation or an imbalance of the nutrient components increases the sensitivity to nematode excreta, so that the traps are formed at a lower nematode concentration in the environment. On the contrary, traps are not produced in rich medium, irrespective of the number of nematodes.

Zootrophic nutrition and chlamydospores. *D. flagrans* is a good model for investigation of the role of zootrophic nutrition. We have previously determined that dilution of liquid medium with water induces the process of chlamydospore formation [10], while it is also one of the requirements for trap formation. Thus, in *D. flagrans* culture grown in a rich medium diluted with water and supplemented with nematodes, two processes are induced simultaneously, i.e., trap formation and chlamydospore development. The traps begin to form by the end of the first day, with their maximum after 2 days of incubation on a shaker at 28°C. Formation and maturation of chlamydospores are completed after 4–6 days of incubation.

The experiments were arranged in order to determine the effect of zootrophic nutrition on the quantity and quality of chlamydospores in the culture. The testing was carried out on two types of cultures. In the first case, the culture was grown for 4 days in 3% sucrose, i.e., under limitation by nitrogen and other nutrients. The second type was a 4-day culture grown in MK balanced medium. Different amounts of the nematodes *P. redivivus* were added to the samples. The same cultures without nematodes, diluted fivefold with water, were used as the control. The content of chlamydospores was determined after 6 days.

After 2 days, numerous traps were formed in all variants, except for the controls. All the nematodes were killed and consumed by the mycelium over 3 days. In culture no. 1 with sucrose excess (control), numerous chlamydospores were budded (Table 3). However, formation of normal chlamydospores was not completed due to the imbalance of the medium. The chlamydospores varied in size and had transparent contents and thin colorless walls, which are characteristics peculiar to immature chlamydospores. Addition of the nematodes (400 individuals/ml) had two significant consequences (Table 3). The yield of chlamydospores was twice as high as in the control. Moreover, the chlamydospores themselves acquired the characteristics of mature spores, i.e., thick dark walls. The substances obtained by the mycelium from the nematodes were evidently used to counteract nutrient deficiency. Further increase in the number of nematodes (samples nos. 3 and 4) did not result in a significant increase of the chlamydospore yield. Numerous immature chlamydospores were budded in the imbalanced sucrose medium prior to the introduction of the nematodes. The result of 2×10^5 chlamydospores/ml probably correlates with the number of chlamydospore buds. Since budding chlamydospores vary greatly in the size and rigidity of their walls, some of them were probably disintegrated during sonication during preparation of the samples, resulting in an underestimate of budding chlamydospores in the control. In this case additional nutrition could not result in a significant increase of the number of chlamydospore initiated prior to the contact with the nematodes. Additional nutrition obtained from the nematode biomass was used for development of chlamydospores and simultaneous mycelial growth. A similar effect was reported for a nematophagous fungus *Arthrobotrys oligospora*, where the addition of a small number of nematodes resulted in a significantly increased conidium yield on solid medium [9].

In the culture grown of the balanced MK medium, dilution with water resulted in formation of normal mature chlamydospores both in the experimental and control variants. Storage compounds in the mycelium had a balanced composition, so that the nematodes were not required for formation of mature chlamydospores. Only an increase in chlamydospore yield was therefore observed (Table 4). An evident correlation exists between the number of nematodes added and an increase of the chlamydospore yield. The coefficient of linear correlation was 0.9 (at a 95% significance level). An average of 19 mature chlamydospores were formed per nematode. Additional nutrition derived from the biomass of the killed nematodes was used to form additional chlamydospores, i.e., the nematode biomass was used to form the reproductive structures of nematophagous fungi. In this case, both processes (formation of chlamydospores and traps) were initiated simultaneously, while, in sucrose medium, chlamydospore buds were formed prior to addition of the nematodes.

Table 4. Chlamydospore yield depending on the amount of nematodes added to *D. flagrans* culture grown in a balanced medium

no.	Nematodes added, individuals/ml	Chlamydospores, 10^4/ml	Chlamydospore yield relative to the control	Average increase in chlamydospore yield, 10^4	Increase in chlamydospore yield per nematode
1	Control, no nematodes	3.4 ± 0.4	1	0	–
2	800	4.7 ± 0.6	1.3	1.3	16.2 ± 2.1
3	1600	6.6 ± 0.5	1.9	3.2	20.0 ± 1.5
4	3200	10.3 ± 0.8	3	6.9	21.6 ± 1.7

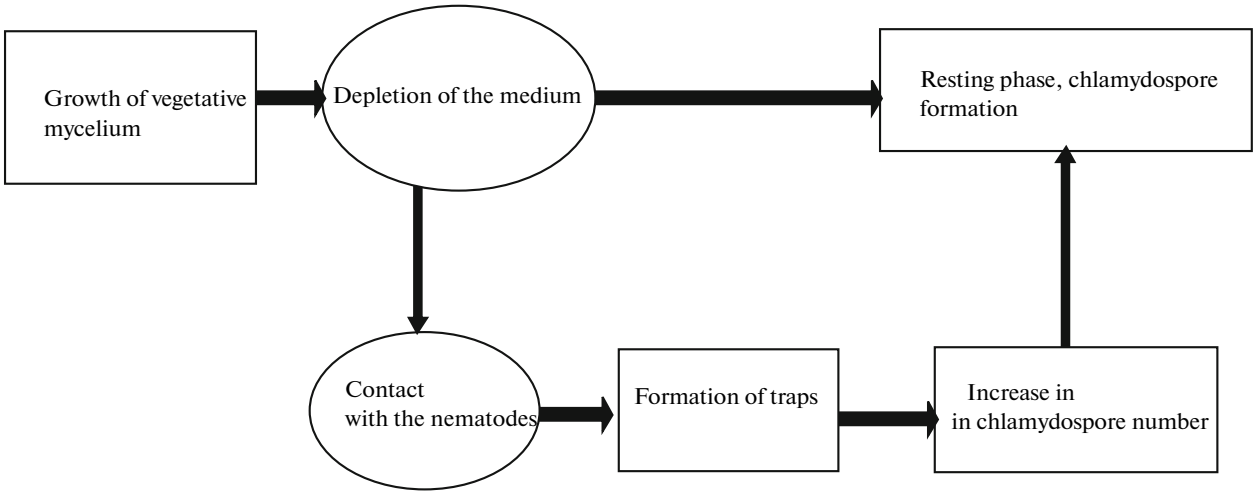
Evolutionary aspect. The role of zootrophic nutrition in the life of carnivorous fungi is still debatable. Two hypotheses exist explaining the reasons for the repeated emergence of carnivorism in the evolution of fungi. The first suggests that nematodes serve as a nitrogen source, since available nitrogen is known to be deficient in plant waste, where carbon sources relatively predominate [11]. The second hypothesis somewhat supplements the first one, suggesting that the nematodes probably act as a source of biologically active compounds, which stimulate sporulation on poor media [9]. In both cases, the limitation by deficient substances is overcome by obtaining them from the nematode biomass.

Our research demonstrated that the *D. flagrans* culture is able to obtain all the nutrient components required for chlamydospore formation from the nematode biomass. The ecological role of the fungus is determined by the state of its mycelium, depending on the growth conditions, and by the conditions in the soil microzone surrounding the mycelium. When there is an excess of organic matter, it utilizes purely saprotrophic nutrition, which is supplemented with the zootrophic mode under nutrient deficiency.

Importantly, unlike addition of soluble carbon and nitrogen sources, addition of the nematodes did not inhibit chlamydospore formation induced by dilution of the medium. The process of chlamydospore formation is not interrupted, and additional nutrients are used for development of additional chlamydospores. Saprotrophic nutrition is therefore the basis of the life cycle of *D. flagrans*, as well as of its ancestors (see figure). For these fungi, zootrophic nutrition is a secondary mode in both evolutionary and ecological aspects, simply supplementing the basic life cycle of a typical saprotroph.

However, when induced, zootrophic nutrition carries out all the functions of nutrition, together with the saprotrophic mode. Singling out the limitation of any specific component as a leading factor in the evolution of carnivorous fungi is a flawed approach. *D. flagrans* is able to form full-grown spores on plant substrates, e.g., on medium with molasses and maize extract. A captured nematode is just another substrate, similar to other organics present in soil. The mycelium extracts all the necessary components from the nematode in order to ensure spore formation and mycelial growth.

Development of the zootrophic mode of nutrition made it possible for carnivorous fungi to expand their ecological niche significantly. The worldwide occurrence of such fungi, including *D. flagrans*, confirms their evolutionary success. The known *D. flagrans* iso-



Role of zootrophic nutrition in the life cycle of *D. flagrans*.

lates are known to be genotypically very close, having diverged from a common ancestor in the relatively recent past (16–23 000 years ago) [12].

The results obtained suggest existence of a complex program for the regulation of zootrophic nutrition in *D. flagrans*. While the mode of nutrition typical of their saprotrophic ancestors remains the main one, the zootrophic mode is used only under conditions of limitation by specific nutrient components. Experiments with submerged culture made it possible to determine the critical concentrations of some carbon and nitrogen sources and compare the sensitivity to the nematode excreta for mycelia grown under different conditions. The nematodes were shown to be used not only to compensate nutrient limitation, but also to ensure formation of full-grown chlamydospores.

ACKNOWLEDGMENTS

This work was supported by the CRDF, grant RBO-11029.

REFERENCES

1. Teplyakova, T.V., *Bioekologicheskie aspekty izucheniya i ispol'zovaniya khishchnykh gribov-gifomitsetov* (Bioecological Aspects of Investigation and Application of Carnivorous Filamentous Fungi), Novosibirsk, 1999.
2. Paraud, C., Hoste, H., Lefrileux, Y., Pommaret, A., Paolini, V., Pors, I., and Chartier, C., Administration of *Duddingtonia flagrans* Chlamydospores to Goats to Control Gastro-Intestinal Nematodes: Dose Trials, *Veterinary Res.*, 2005, vol. 36, no. 2, pp. 157–166.
3. Grõnvold, J., Nansen, P., Henriksen, S.A., Larsen, M., Wolstrup, J., Bresciani, J., Rawat, H., and Fribert, L., Induction of Traps by *Ostertagia ostertagi* Larvae, Chlamydospore Production and Growth Rate in the Nematode-Trapping Fungus *Duddingtonia flagrans, J. Helminthol.*, 1996, vol. 70, no. 4, pp. 291–297.
4. Gronvold, J., Wolstrup, J., Nansen, P., Larsen, M., Henriksen, S.A., Bjorn, H., Kirchheiner, K., and Kristiansen, H.L., Biotic and Abiotic Factors Influencing Growth Rate and Production of Traps by the Nematode-Trapping Fungus *Duddingtonia flagrans* when Induced by *Cooperia oncophora* Larvae, *J. Helminthol.*, 1999, vol. 73, no. 2, pp. 129–136.
5. Friman, E., Olsson, S., and Nordbring-Hertz, B., Heavy Trap Formation by *Arthrobotrys oligospora* in Liquid Culture, *FEMS Microbiol. Lett.*, 1985, vol. 31, no. 1, pp. 17–21.
6. Nordbring-Hertz, B. and Stõlhammar-Carlemalm, M., Capture of Nematodes by *Arthrobotrys oligospora*, an Electron Microscope Study, *Can. J. Bot.*, 1978, vol. 6, no. 10, pp. 1297–1307.
7. Scholler, M. and Rubner, A., Predacious Activity of the Nematode-Destroying Fungus *Arthrobotrys oligospora* in Dependence of the Medium Composition, *Microbiol. Res.*, 1994, vol. 149, pp. 145–149.
8. Semenov, S.M., *Laboratornye sredy dlya aktinomitsetov i gribov* (Laboratory Media for Actinomycetes and Fungi), Moscow: VO Agropromizdat, 1990.
9. Migunova, V.D. and Byzov, B.A., Determinants of Trophic Modes of the Nematophagous Fungus *Arthrobotrys oligospora* Interacting with Bacterivorous Nematode *Caenorhabditis elegans, Pedobiologia*, 2005, vol. 49, no. 2, pp. 101–108.
10. Anan'ko, G.G. and Teplyakova, T.V., Biotechnology for Production of a Preparation against the Nematode Parasites of Cultured Plants, in *Dostizheniya sovremennoi biotekhnologii* (Progress in Modern Biotechnology), Drozdov, I.G., Ed., Novosibirsk, 2008, pp. 328–335.
11. Jaffee, B.A., Wood, Nematodes, and the Nematode-Trapping Fungus *Arthrobotrys oligospora, Soil Biol. Biochem.*, 2004, vol. 36, no. 7, pp. 1171–1178.
12. Ahren, D., Faedo, M., Rajashekar, B., and Tunlid, A., Low Genetic Diversity among Isolates of the Nematode-Trapping Fungus *Duddingtonia flagrans*: Evidence for Recent Worldwide Dispersion from a Single Common Ancestor, *Mycological Res.*, 2004, no. 10, pp. 1205–1214.