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FUNGI go HUNTING



Fungi are most amazing creatures among the higher organisms; combining the traits of plants and animals, they form a separate large kingdom. These microscopic organisms are ubiquitous and wonderfully diverse, predatory fungi being the unique ecological group of them. During the evolution, these minute predators had acquired manifold hunting tools for trapping their victims, roundworms (nematodes), which include numerous parasitic species dangerous for the health of plants, animals, and even humans. Predatory fungi may become an efficient and ecologically safe alternative to the currently available expensive and highly toxic antihelminthic preparations, which typically lead to environmental pollution and elevate the toxin resistance of the parasites themselves



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Photo shows larvae of the potato nematode in the sticky nets of predatory fungus *Duddingtonia flagrans*; top, hyphae (threads of the mycelium) of the predatory fungi cultivated in agar medium

Key words: predatory (nematophagous) fungi, parasitic nematodes, traps for nematodes, chlamydospores, biopreparation

Today, the plant helminthiasis caused by roundworms (*nematodes*) present a serious challenge for both large agricultural plants and common gardeners: according to the expert estimation, phytoparasitic nematodes “consume” up to 10 % of the world yield!

The so-called *root-knot nematodes* (*Meloidogyne incognita*, *M. hapla*, etc.), widespread in open field and greenhouses, are especially deleterious. These parasites induce the root tumors (*galls*), which can almost halve the yield. These nematodes affect many cultures, from the tomato and cucumber to melon and ginseng. Other parasitic nematodes are also able to cause a considerable damage, for example, the stem eelworm (*Anguillulina dipsaci*) affects the strawberry, and the golden nematode (*Globodera rostochiensis*) is attracted by the potato, the “second bread” in Siberia.

Both climate changes and expansion of trade relations between regions have allowed many parasites, phytoparasitic nematodes (earlier regarded as a quarantine object) included, to successfully colonize new areas. Illustrative examples for Siberians are not only the notorious Colorado potato beetle, first recorded in the Novosibirsk oblast in 1978, but also the above-mentioned potato nematode, a malicious rapidly propagating pest, already found in many areas of the oblast.

The pets and agricultural animals are affected by parasitic nematodes not to a lesser degree. Note that several animal helminths represent a serious menace for

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IN THE WORLD OF NEMATODES

The roundworms, or nematodes (Lat. *Nematodes*), display tremendous systematic and ecological diversities. Currently, this group comprises about 80 000 species; however, their number may reach a million according to some estimations. Among the nematodes, one can find diminutive creatures with a body length of less than 100 microns as well as giants, such as the nematode parasitizing the sperm whale placenta, with a length of 8 meters. Just a detail: an adult female roundworm can daily lay up to quarter million eggs!

Roundworms have successfully colonized almost all known habitats, air included: they are present in fresh water bodies, ocean depths, soil, and living bodies themselves. The last type of nematodes, that is, parasitic nematodes, are only part of the multitude of numerous free-living species (1 dm³ of the soil surface layer may house up to two million individuals).

Round worms live at the expense of plants and animals; human is one of their hosts, about fifty nematode species are able to infest humans. Typically, nematodes live in cavernous organs connected with outside (gastrointestinal tract, lungs, and kidneys) as well as in the connective and lymphatic tissues. Currently, nematodes are most abundant among the agents of helminthiasis with a developmental phase in soil. For example, according to the WHO data, the annual morbidity of ascariasis in the world exceeds one billion people and those of ancylostomiasis and trichocephaliasis are slightly lower



Root-knot nematodes are abundant in both greenhouses and open fields. Entering the roots of cucumber, tomato, and other plants, they induce development of tumors on them, interfering with their metabolism. The affected plants droop, become susceptible to diseases, and die. The golden potato nematode damages potato roots forming cysts; these round structures are female individuals; their integuments are transformed into rigid envelope resistant to environmental impacts; and they are filled with eggs and the larvae developing from them. *Left*, cysts on a potato root; *top*, cucumber root knots

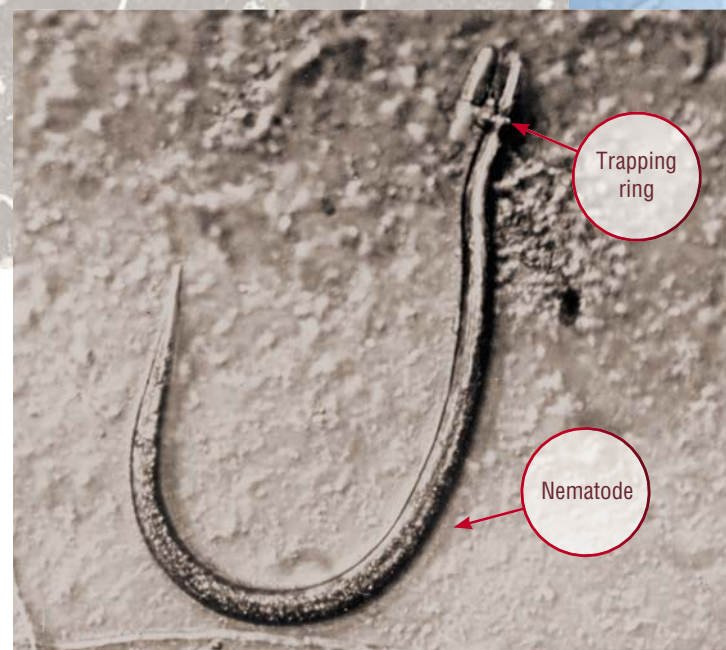
Over 100 species of the predatory filamentous fungi are known so far. These microscopic soil dwellers have been found in all parts of the world and all climatic zones. Utilizing a tremendous mass of soil nematodes, they play a significant role in the total turnover of carbon, nitrogen, and other most important biogenic elements

human health as well; they can infect humans either by a direct contact or via soil from pastures. A considerable infection rate of dogs and cats contributes to an increase in the morbidity among human population with rather serious consequences. Note here that according to some estimation about quarter of the world population is infected with parasitic nematodes (pinworms, ascarids, and so on).

In ambush for worm

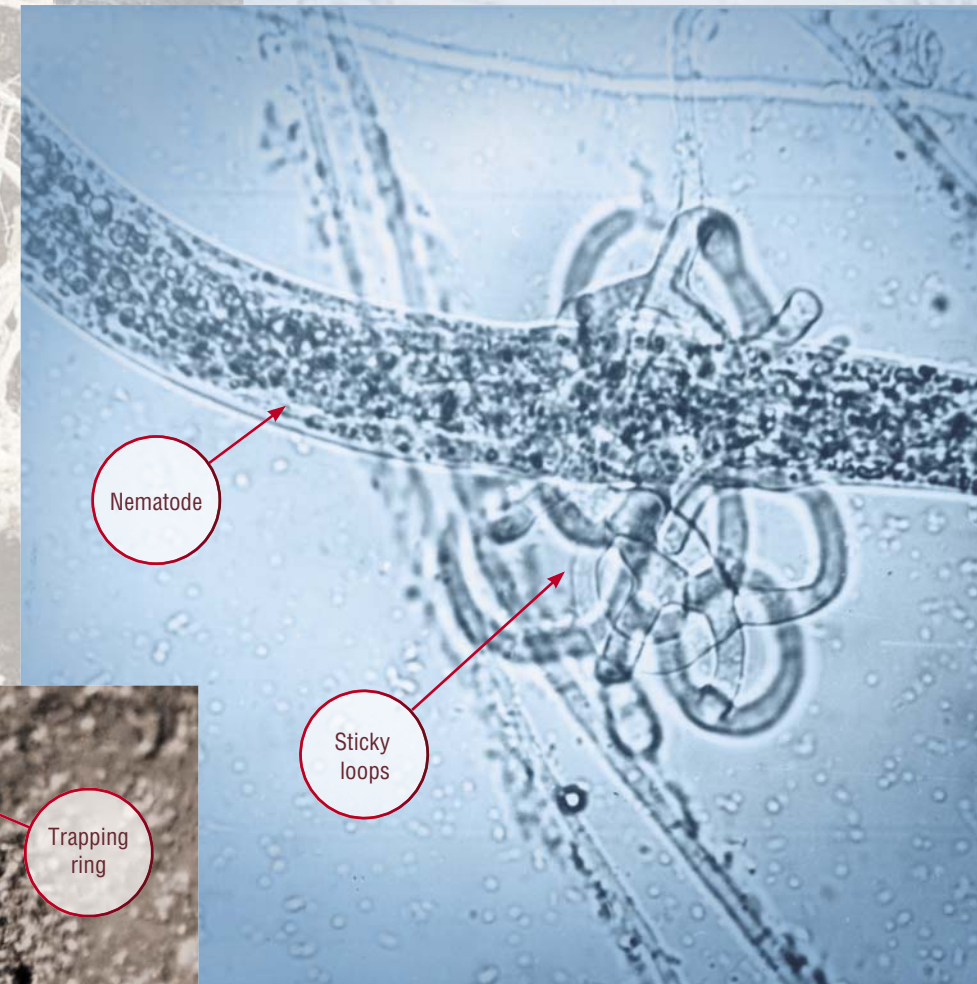
Of special interest among the natural regulators for nematode population size are *predatory fungi*.

The term “predatory” applied to a fungus may seem rather strange, the more so since unlike the large fungi, *macromycetes*, which unite all the



The hunting tools of predatory fungi are rather diverse, including contracting rings, sticky loops, and even “chemical weapons”.

Photo shows a successfully caught prey: a soil nematode constricted with a trapping ring of the fungus *Dactylariopsis brochopaga* (above) and a nematode trapped into sticky loops of the fungus *Arthrobotrys compacta* (top right). *Light microscopy*

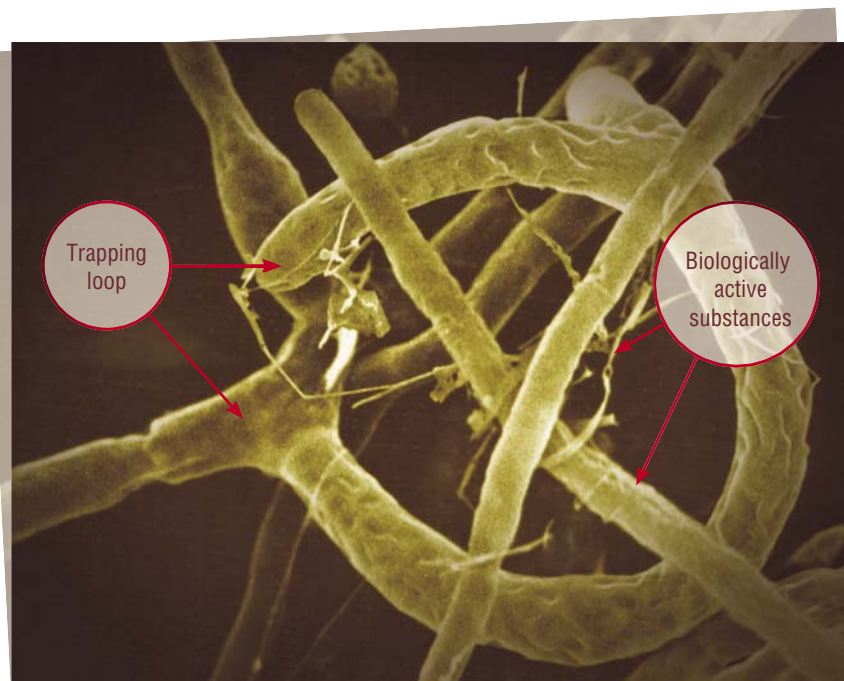


edible fungi, the predatory fungi are *micromycetes* and are represented by finest spider threads (*hyphae*) visible only with the aid of a microscope.

The specialized traps capable of catching actively moving nematodes with their body diameter several hundredfold larger as compared with the diameter of fungal hyphae are formed on these ultrathin threads. Moreover, the types of these trapping tools filled with “chemical weapons” are most diverse, including sticky loops, warheads, and contracting rings.

The research into predatory fungi aiming to find the strains promising as producers of antinematode drugs against was started at the Siberian Institute of Agriculture and Chemicalization as early as 1971.

No more than one hundred specialists worldwide were involved in this problem at that time. The experimental data on nematophagous activity of predatory fungi obtained by that time were sparse and contradictory, casting doubts on feasibility of their application to control nematodes. Therefore, scientific justification for an efficient use of predatory



PREDATOR'S TACTICS AND STRATEGY

Independently of the type of hunting tools, the mechanism of fungal predation includes release of attractants and toxic compounds; penetration of fungal hyphae into the body of a paralyzed nematode; and secretion of enzymes and antibiotics for “digesting” the nematode contents and inhibiting microbial competitors.

All these biologically active compounds are water-insoluble and thus avoid a rapid degradation by soil microorganisms. In particular, these compounds include the toxins of terpene nature contained in the adhesive substance secreted on the surface of traps and mycelium (Radzhabova, 1971; Bekker, 1972).

It has been earlier assumed that dissolution of the external worm integuments by the enzymes released by fungi accelerates penetration of fungal hyphae into the nematode body. However, it is well known now that fungal hyphae move apart the nematode cuticle with the help of specialized fiber structures (Teplyakova and Ryabchikova, 1991). This is also suggested by an elevated calcium concentration in the fungal traps, detected using X-ray microanalysis. The calcium content is especially high (increased 30- to 40-fold) in the trapping rings of fungal mycelium.

As is known, calcium plays an important role in the mechanism of animal muscle contraction, being involved in the regulation of actomyosin (contractile muscle protein) function. Presumably, an analogous situation takes place in the world of fungi, the biochemistry of which has much in common with animals (Bekker, 1972). Predatory fungi may use such an actomyosin system for different purposes: for contracting their trapping rings or squeezing out toxic sticky substances to the surface of trapping loops.

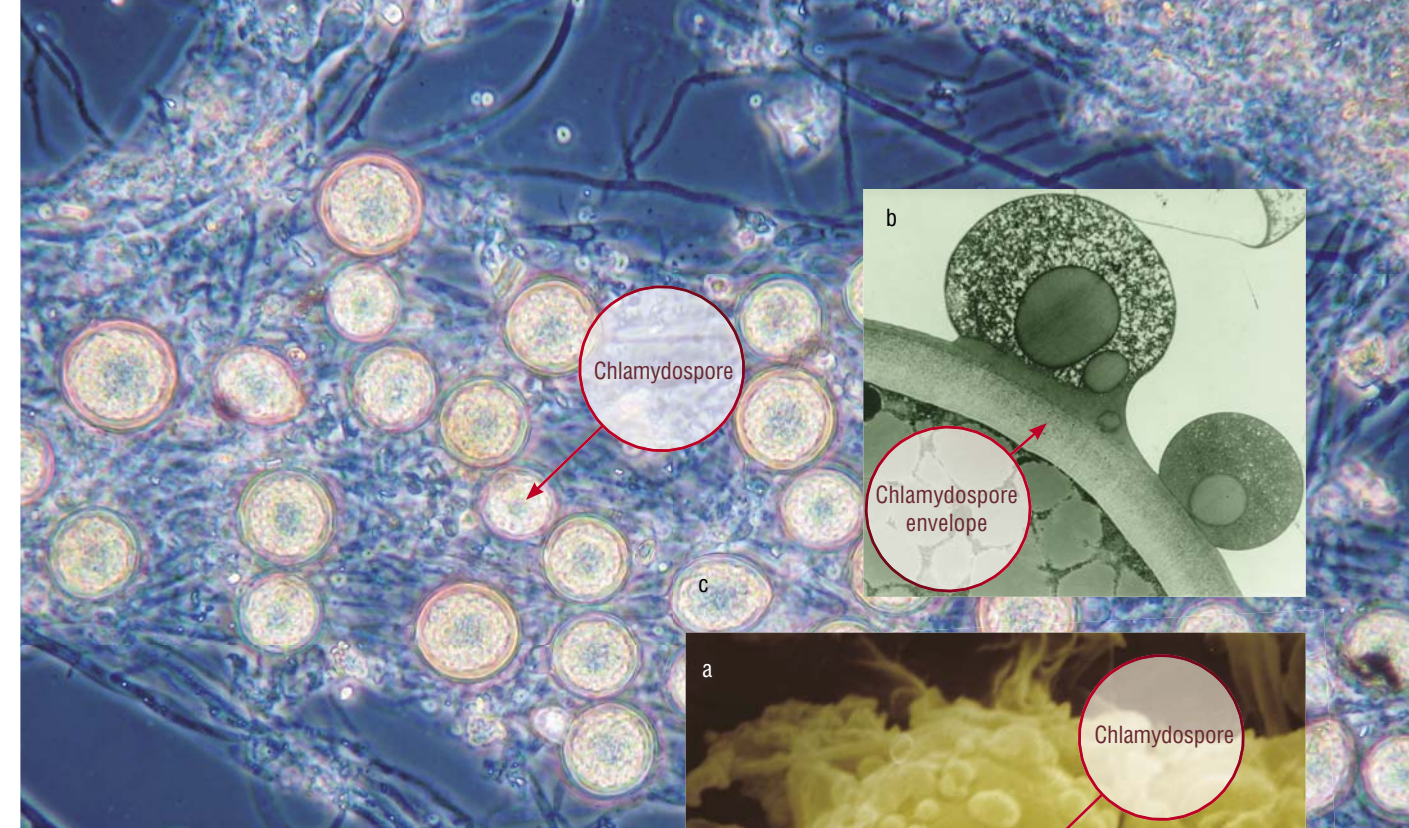
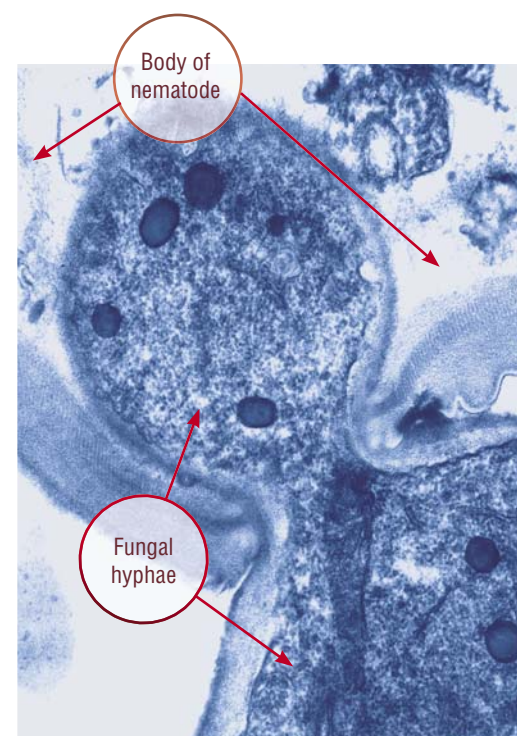
When a biopreparation involving predatory fungi is added to soil, the studied strains develop the maximal number of trapping organs on day 14. Therefore, it is most purposeful to apply such a preparation at least 2—3 weeks before planting (Soprunov, 1958; Teplyakova, 1999)

fungi in biological protection required manifold studies of their life activities, including the life cycles, specific behavioral features, and interactions with other microorganisms in both soil and cultures.

A deeper insight into the very mechanism of predation was also necessary as well as substantiation of the methods for their selection and stabilization of effective strains. It was impossible to elaborate biotechnology for the antinematode preparation and the recommendations for its practical application without answering all these questions.

As early as 30 min after trapping a nematode with the help of sticky trapping loops (left), hyphae of the fungus *Arthrobotrys compacta* move apart the integuments to enter the body of a paralyzed roundworm (below).

Scanning and light microscopy



Behind the walls of chlamydospore

When isolating predatory fungi from soil, many researchers observed development of *chlamydospores*, large structures with thick walls, on the fungal mycelium; these chlamydospores disappeared with further replatings. As was considered, chlamydospores were formed only under adverse conditions and their role in the fungal life cycle was rather vague. However, it has been shown that chlamydospores are the most important and even, in certain cases, the major life form for the predatory fungi (Teplyakova, 1999).

This discovery explains why it is so difficult to isolate predatory fungi from soil using commonly accepted microbiological methods. Indeed, the saprophytic fungi at vegetative developmental stages predominantly develop on nutrient agar, whereas the chlamydospores of predatory fungi require stimulation for their germination. Thus, a special technique is used to isolate nematophagous fungi: either pieces of soil containing its inhabitants, nematodes, are placed onto the agar surface or nematode laboratory culture is added.

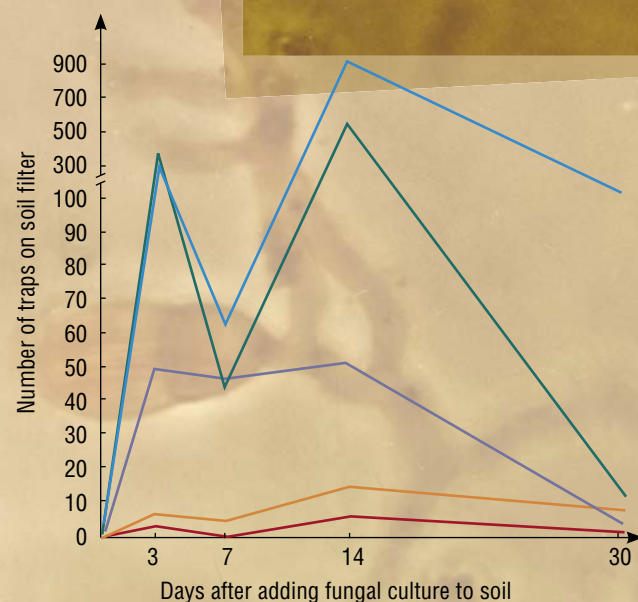
The metabolites secreted by worms stimulate chlamydospores to germinate, and trapping tools are formed on the fungal hyphae. Just in one week, seen on the agar surface are real “slaughters”, that is, the areas filled with dead nematodes and fungal hyphae with traps, as well as the asexual reproductive organs, *conidiophores*, with *conidia* (fungal spores) or conidial chains. During further cultivation on nutrient agar, fungi gradually lose their ability to

The fungi are distinguished by their complex life cycles and variety of reproduction methods. The most important life form for the predatory fungi is chlamydospore – survival structure of predator covered by the thick envelope which serves as a vegetative analogue of the true conidium seeds.

Photo shows: external view (a) and fragment of internal structure (b) of chlamydospore *Duddingtonia flagras* (c). Light and electron microscopy

form chlamydospores and commence growing prevalently as a vegetative mycelium.

The lack of knowledge about these specific features could lead to mismatch between experimental and field data. Indeed, the biopreparation grown in grain nutrient media with the fungal mycelium bearing conidia as the major component was most frequently added to soil. However, such a strain in soil emerged to be insufficiently viable; correspondingly, a preparation highly active under laboratory conditions appeared inefficient in the field.



It has been shown by the example of a predatory nematophagous fungus belonging to the genus *Arthrobotrys* that the laboratory culture added to soil predominantly develops chlamydospores. They germinate and form traps when nematode larvae appear nearby. After the “feast”, the next generation of chlamydospores is produced. This cycle will be repeated until helminths are present in the soil.

Left, the dynamics of hunting efficiency of laboratory cultures of five *Arthrobotrys* strains under natural conditions assessed according to the intensity of trap formation on fungal mycelium

The medication for soil

The search of natural populations for predatory fungi has allowed the efficient strains with a high nematophagous activity to be obtained.

These strains are *Arthrobotrys oligospora* 3062D (USSR author’s certificate no. 1688818, 1991), which was used to elaborate the technology for producing the biopreparation Nematofagin-BL, approved for use in Russia, and *Duddingtonia flagrans* F-882 (patent of the Russian Federation no. 2253671, 2005), displaying a high ability to develop chlamydospores in culture.

It has become evident that depending on the final goal, it is possible to produce both liquid and dry biopreparations using different technological procedures.

When a dry preparation containing chlamydospores is intended for adding to soil, it may be produced using various carriers, for example, a layered mineral, vermiculite (patent



of the Russian Federation no. 2366178, 2009). In a simpler case, the preparation is produced on grain media and used for therapy and prevention of parasitic nematodes infecting cattle or pets by administering it as a feed.

Liquid preparation may be produced using the already existing facilities for manufacture of bacterial preparations for plant protection; the more so since inexpensive media have been already selected for the above mentioned strains as well as the cultivation conditions.

In particular, over 5 tons of the liquid preparation with *Duddingtonia flagrans* F-882 strain as a major component intended for pilot trials in greenhouses were produced by the Berdsk plant, limited liability production company OOO OP *Sibbiofarm*. Note that the fungal biomass produced in such fermenters contains a wide range of biologically active substances. The recent studies at the State Research Center of Virology and Biotechnology Vector in collaboration with virologists demonstrate also an antiviral activity of these metabolites of nematophagous fungi (patent pending no. 2011110830 of March 22, 2011).

Trials of various forms of this biopreparation performed in Russia (Novosibirsk oblast, Kemerovo oblast, Altai krai, and Krasnodar krai) using different agricultural plants (cucumber, tomato, potato, strawberry, and others) have demonstrated:

- a decrease in the soil infection with nematodes in greenhouses by 86 % and the resulting increase in cucumber yield of 2 kg per 1 m² and more;
- a decrease in infection with the potato nematode in the susceptible cultivars by 52–70 % and the resulting 1.5–2-fold increase in their yields;

Tests of liquid and dry variants of the biopreparation with predatory fungi have demonstrated their high efficiency for both greenhouses and field soil. The control plants (soil without the biopreparation) displayed a considerably delayed growth and lower yield

- a 2–17-fold decrease in the counts of stem nematodes in strawberry leaves depending on the preparation dose and form with a concurrent increase in the share of large berries by 5–17 %;
- a stimulatory effect on the plant growth and development ranging from flower plantlets to pine seedlings.

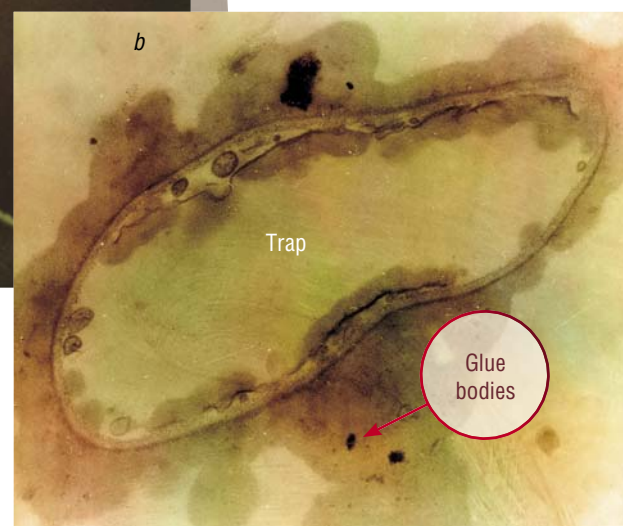
A production experiment with the liquid biopreparation was conducted in the winter of 2005–2006 at the state greenhouse plant Sukhovskii (Kemerovo oblast) aiming to confirm its long-term effect observed earlier (Tepliyakova, 1999; Tepliyakova *et al.*, 2008). It has emerged that the yield of the cucumber hybrid cultivar Effekt during the first year when the preparation was used increased by 0.1–1.0 kg/ m² and by 0.6–0.7 and 1.3–2.8 kg/m², respectively, in the next two years (without any additional application of the preparation).

From mice to red deer

It is known that the population of parasitic nematodes in pastures contains 95 % of invasive larvae, which rapidly nullifies the effect of antihelminthic therapy and contributes to an increase in expenditures making necessary a repeated antiparasitic treatment (Herd, 1985). As is currently ac-



A high nematophagous effect of the dry biopreparation of predatory fungi, rich for chlamydospores, is determined by numerous traps formed in soil. In the case of a liquid variant, containing only fungal mycelium, nematodes are killed by the toxic compounds secreted by fungal hyphae.
(a, b) Adhesive and biologically active substance secreted by the predatory fungus *Arthrobotrys oligospora*.
Scanning and transmission electron microscopy



cepted, the optimization of antiparasitic efforts implies, first, eradication of helminths in the animal body with the help of antihelminthic drugs and, second, involvement of a number of measures that limit the counts of larvae and eggs in environment.

One of the promising methods for controlling the parasitic nematode larval population in environment is biological control with the help of predatory helminthophagous fungi. This approach was tested in laboratory experiments in collaboration with the Institute of Veterinary (Siberian Branch, Russian Academy of Agricultural Sciences) aimed to assess the nematophagous activity of the *D. flagrans* F-882 strain towards the sheep, red deer, and horse nematodes. As has been shown, this strain kills “in vitro” 93–98% of the parasitic nematodes belonging to the genera *Trichonema*, *Strongylus*, *Alfortia*, and others (Teplyakova and Efremova, 2005; Efremova and Teplyakova, 2007; Efremova *et al.*, 2007).

The experiments with white laboratory mice fed with the grain biopreparation involving the strain in question have demonstrated that the fungus retained its viability

and nematophagous activity having passed through the animal digestive system. The hyphae with trapping loops and chlamydospores were detectable in animal feces, while the helminths “washed out” of the mouse gastrointestinal tract displayed considerably injured cell structures in all tissues and organs as compared with the control, which eventually led to their death. Presumably, these injuries were caused by the substances produced by fungal cells, since any signs of a “direct” infection of nematodes by fungi were undetectable.



This predatory fungus, *Duddingtonia flagrans*, succeeded in trapping a nematode parasitizing agricultural and wild animals: sheep, horse, and red deer

Toxicological and hygienic estimations of the predatory fungal cultures at the Angarsk Institute of Occupational Medicine and Human Ecology, which is certified by the State Committee of the Russian Federation for Standardization and Metrology, has demonstrated that the fungal cultures have neither general toxic nor pathogenic effects on the organism of warm-blooded animals independently of the route they entered the body. The fungi do not reproduce in the body, do not cause any infectious process, and are rapidly eliminated.

All these data suggest that predatory fungi can be used for therapy and prevention of helminthiasis (also dangerous for humans) in animals. Since the fungal chlamydospores retain their viability after passing through the gastrointestinal tract, the predatory fungi continue their “work” in feces, thereby healing not only animals, but also environment.

As for the antihelminthic preparations for humans, a promising direction here is extraction of active low-toxic natural compounds from the biomass of predatory fungi, any quantities of which can be produced using the already developed protocols. In order to elevate the efficiency and decrease the toxicity of such natural anthelmintics, they can be encapsulated into vehicle structures of a liposome type, which will allow for a decrease in their dosage.

Photos by the courtesy of the author
and E. I. Ryabchikova (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Novosibirsk)

In the industrially developed countries, the field of biotechnology associated with biological protection developments is a priority for a good reason: each dollar invested to this field brings up to 150% profit (Yatsenko, 2008). The market of microbiological biopesticides grows very rapidly, having increased almost fivefold in the United States over the last two decades.

However, the share of biological protection tools as compared with chemical ones still remains rather small, especially in the countries with intensive farming. That is why the domestic biotechnological developments that immediately applicable in plant breeding and veterinary are of great importance.

By the way, it has been recently found out that the biologically active compounds of the nematophagous fungus *D. flagrans* are active against HIV, type A influenza virus, and vaccinia virus. All this suggests that these amazing creatures, predatory fungi, will repeatedly surprise their researchers.

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