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Carnivorous fungi application for pesticide-free vegetable cultivation

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ABSTRACT

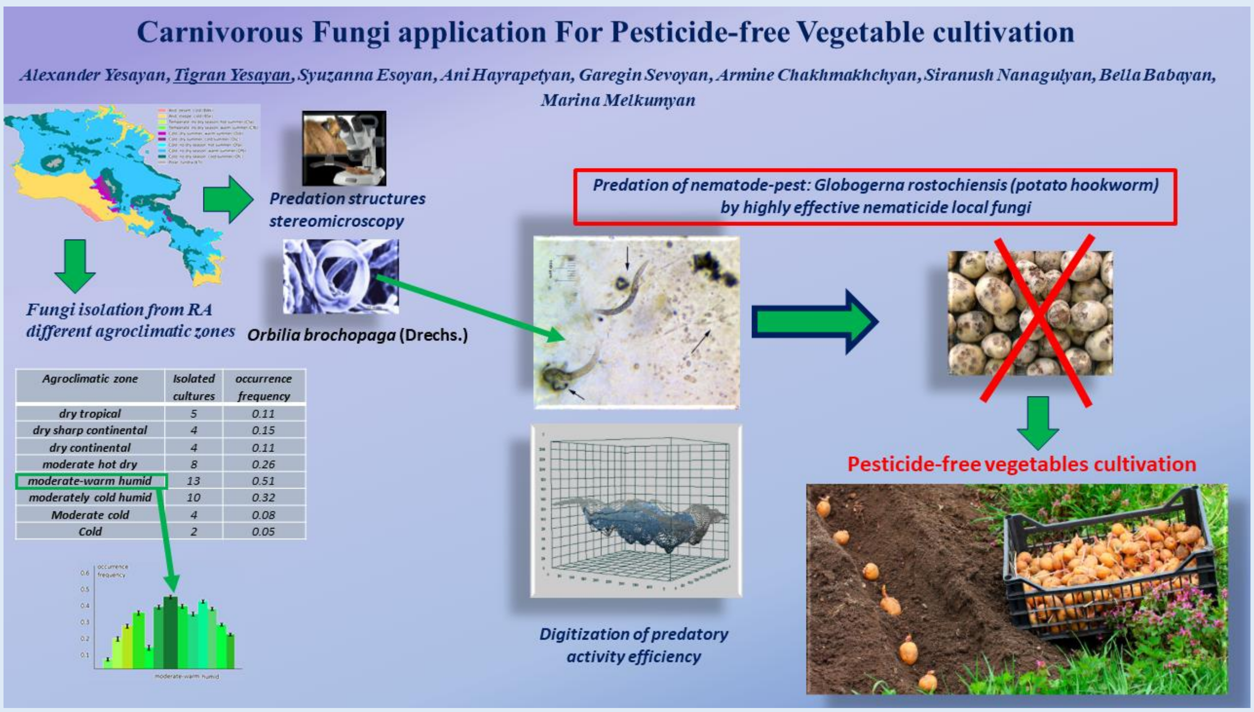
Background: Carnivorous fungi are commonly found inagricultural soils around the world, including RA. These fungi are trapping and eating microscopic nematode worms that reside in the soil and water. Phytopathogenic nematodes are harmful for agriculture, significantly decreasing the quality of vegetables and other crops. The classical pesticides are either ineffective or unsafe for human health and environment. Thus, an application of innovative biopreparations that address these safety and efficacy concerns are more preferable in terms of green agriculture and pesticide free food production.

Objectives: This ecological and genetical study of Armenian carnivorous fungi, includingthe isolation, identification and *in vitro* study of nematicide activity against the local nematode pests, can be prospective for obtaining pesticide-free vegetables.

Results: In this study, 50 strains of predatory fungi were isolated. According to the obtained data, local predatory fungi isolated from Armenian soils have demonstrated higher activity against both vinegar eel (*Turbatrix aceti* test object) and the local species of potato hookworm (*Globodera rostochiensis*) compared to the analogous trade preparations.

Conclusions: The collected data demonstrated that different species of carnivorous fungi are present in Armenian soil. Particularly, the studied *Orbilia oligospora* (Fresen.) Baral and E. Weber (syn. *Arthrobotrys oligospora* (Fresen.) and *Orbilia brochopaga* Drechsler species of fungi had shown high activity against the potato hookworm. The identified fungal strains have demonstrated more efficiency, than the fungi-based trade biopesticides. Probably it is related to the features of source of pathogen and fungi isolation, which defines the strain-specificity of nematicide enzymes of the observed fungi.

Keywords: nematophagous fungi, pesticide-free vegetables, nematode toxins, fungal nematicide enzymes, *Orbilia oligospora, Orbilia brochopaga,* potato hookworm



INTRODUCTION

Protecting plants is one of the most important problems in agriculture. It is directly related to the quality of vegetables and fruits which are important as the main sources of most vitamins. There are various pests that can enter plants and agricultural crops, including phytopathogenic microorganisms (bacteria, fungi), phytophagous insects, worms, etc. [1]. Particularly, the mentioned phytopathogenic nematode worms are common for Armenia and other countries worldwide. There are more than 3000 species of phytopathogenic nematodes [2, 3]. These worms are able to significantly harm agriculture and cause an average 10% yield decrease in crop production, which can result in approximately 120 billion economic losses worldwide [4]. Phytopathogenic nematodes are particularly harmful for most crops, such as coniferous trees and other plants. Especially, phytopathogen nematodes present a significant danger for potatos and other vegetables. They are able to destroy the plant root system, gnaw through the tubers of vegetables, and harm the fruits. In these regards, they have both direct and indirect negative impact on vegetable and fruit food products quality [5].

The direct influence of phytopathogenic nematodes is caused by the contamination of food products by the nematode-derived toxins and other bioactive compounds, such as biomolecules that are being produced by the plant for protection from nematodes. All the mentioned biomolecules can alter human metabolism with potentially negative consequences for health. The presence of phytopathogenic nematodes can contribute to damage of the human digestive system, intoxications, and allergic reactions. The indirect harm of phytopathogenic nematodes is caused by the secondary infection of the damaged regions of vegetables by pathogenic bacteria and fungi. As a result, the vegetables could be contaminated by the metabolites of secondary infection agents: bacteria, fungi, etc. Some of these infection agents can synthesize toxins, such as aflatoxins, whichis extremely harmful.

Phytopathogenic nematodes can synthesize a wide diversity of biologically active compounds with versatile mechanisms of activity. They synthesize the following compounds: cellulases, xylanases, pectinases, proteases, cysteine protease-like protein, calreticulin, avenacins, effector proteins, glycoproteins, and glucanases. These compounds undergothe host-plant infection process, resembling enzymes that decompose the plant cell wall. Nematodes can also activate Reactive Oxygen Species (ROS) synthesis in plant cells, which leads to free radical-mediated destruction in plant cells. Consistent consumption of food products contaminated by the mentioned compounds can lead to the development of chronic diseases (gastritis, colitis, dermatitis, allergies, etc.) and possibly oncological pathologies [7,8,9].

Various methods are being applied for plant protection, including chemical andphysical methods, such as such as the cultivation of plants using =Genetically Modified Organisms (GMO) technologies, which are resistant to some nematode species. However, the majority of methods have their disadvantages and hidden risks, including the significant decrease in quality of vegetable foods, direct (the classical chemical pesticides toxicity, genotoxicity, etc.) and indirect negative impact on both environment and the human health (pesticide accumulation in waters and soil, genetical changes in crops biodiversity due to the uncontrolled spread of GMO plants pollen, etc.). Most active components of chemical nematicide preparations, regardless of the country of their origin, are highly toxic for both human and animals [10,11]. Plant-parasitic nematodes are a possible cause of many global economic problems, resulting in billion-dollar annual losses due to poor crop yields. The number of the registered nematicide preparations has declined substantially over the last 25 years due to concerns about their non-specific mechanisms of action, potential toxicity for both human and animals, and likelihood to cause environmental damage [12, 13].

In these regards, the application of biological methods of plant protection is more desirable because of low toxicity risks. Nematophagous fungi as the biocontrol agent against the worm pest may be considered a possible solution to harmful pesticide use. The mentioned nematophagous fungi are representatives of carnivorous fungi. They include about 200 species, which can be isolated from different geographical zonesMostly they include representatives of *Ascomycota, Mucoromycota*, and *Basidiomycota* [14-16]. Carnivorous fungi have different classifications compared to other ecological groups of parasitic and scavenging fungi.For example, fungi that colonize the epidermis, nails, hair, skin, scales or feathers of living birds and other animals or animal corpse are considered to be dermatophytes rather than predaceous. Similarly, the fungal species, identified in orifices and the digestive tract of various animals are not carnivorous. On the other hand, insect pathogens that stun and colonize insects are normally labelled carnivorous if the fungal thallus is mainly in the insect (e.g. *Cordyceps*) or if it clings to the insect (e.g. *Laboulbeniales*) [17]. The main characteristic features of carnivorous fungi are their predaceous lifestyle and ability to consume some types of animals, such as worms by forming the specific traps. The fungi traps, made ofalterations of mycelium, fix, paralyze, and immobilize the victim by penetrating its body with a mycelial hyphae. . There are two general mechanisms of trapping, observed in nematophagous carnivorous fungi: the constricting rings (active traps) and the adhesive structures (passive traps) [18,19]. According to the data of recent years research, DNA Sequencing of ribosomal genes of carnivorous fungi has shown that the mentioned types of traps occur in separate fungus lineages, as an example of convergent evolution process [20].

Nematophagous fungi and/or endophytic fungi can directly attack, kill, immobilize, or repel nematodes, confuse nematodes finding their host, interfere with giant cell development, compete for resources, or use a combination of these options. These fungi can also capture, parasitize, or paralyze nematodes,acting as natural enemies of plant and animal-parasitic nematodes. They are divided into four groups: endoparasitic fungi, nematode-trapping fungi (NTF), opportunistic fungi, and toxin-producing fungi. NTFs are a unique group of soil-inhabiting fungi that can switch from the saprophytic to pathogenic lifestyle once they come in contact with nematodes as an adaptationto nutrient depletion [21, 22].

Carnivorous fungi are potentially harmless for humans,fishes, and other animals. The various species and genera representatives of nematophagous fungi are able to synthesize various secondary metabolites. They have the versatile biological effect, including nematocidal, insecticidal, bactericide, fungistatic and fungicideFor example, there are several nematicide compounds that exhibit antimicrobial properties, such as hirsutellins, ophiobolins, pochonins, drechslerins, lecanicillins, lilacins, in addition to the insecticidal compounds destruxins, 6-pentyl-α-pyrone, harzianolide, andsome antimicrobial extracellular secretion proteases.Thus, the mentioned compounds can be potentially used as natural antimicrobials, andincrease the functionality of food products produced without antibiotics and other artificial antimicrobials agents [23, 24]. Carnivorous fungi can potentially be used in agriculture as the active component of bio-preparations, to reduce the number of agricultural nematode-pests [25]. According to well established research, the maximal efficiency of biopreparation (which consist of fungal spores (biomaterial) in dry condition or in the form of water suspension) depends to chemical consistency, pH and the microbiome of that particular soil and water. Thus, studies elaborating preparations using nematophagous fungi isolated from a particular geographic region is needed [26].

Current research is devoted to the observation and study of carnivorous fungi in different regions of Armenia with their potential application in agriculture against the plant's pests.

MATERIALS AND METHODS

Fungi and nematodes sample collection, isolation and cultivation: At the first step of research, the regions of RA for the sample collection were chosen in accordance to the intensity of agriculture there. The samples of soil with the residues of plant detritus were collected in sterile plastic containers. All the experiments, starting from the samples processing were carried out immediately after the samples collection. After the experiment, the storage of samples was carried out at 4 °C, according to the generally accepted methods [27,28].

The cultivation of nematodes was carried out in 90 mm Petri dishes on solid starvation agar media (according to the cultivation 3 g agar and 100 mL water, autoclaved within 20 min at 1 atm, pH 6-7) in aerobic conditions in room temperature for a 48 h. he isolation of fungi was carried out using the direct soil-scattering method. For that purpose, 1 g of soil samples from each the studied region were sprinkled directly onto 2% water agar plates, ensuring the appropriate density of soil and its uniform distribution on a surface of media and incubation of plates at 28 °C. Daily observations of the predation presence was carried out by the optical microscopy method. After the seventh days of observations incubation, the trapped nematodes were visible as surrounded by each fungus-produced ring-traps. After that the fungal conidia were growing around the deceased nematode bodies. The spore producing structures are growing upright and produce the conidia in region of apices. For the purpose to pick the fungal hyphae and/or spores in the vicinity of predated nematodes and further transfer of them to Potato Dextrose Agar (PDA) plates, the sterilized needles were applied with 3-5 purifications. Then the purified fungal strains were stored at 4 °C [29,30].

Morphological observations of carnivorous fungi: For the isolation and identification of carnivorous fungi the generally accepted microscopy methods were applied to the study of morphological characteristics of fungi [31, 32]. All the morphological observations were carried out by the application of stereomicroscopy and photography methods using VHX-7000 digital microscope, Keyence (produced in Japan), optical microscopy using Axio Scope 5, Carl Zeiss Suzhou Co., Ltd., Suzhou, (produced in China), equipped with an Axio Cam 208 color camera (produced in China).

The assessment of *in vitro* predatory activity of fungi: The predatory activity screening was carried out for the further evaluation of fungal efficiency as nematicide factor. The predation activity of all the isolated fungi was assessed by the application of nematodes, which were isolated from the studied regions of RA: *Globodera rostochiensis* potato hookworm and *Turbatrix aceti* vinegar eel (vinegar nematode or so called *Anguillula aceti*) [33]. Fungal strains were cultured on PDA plates within a period of 5 days. Then from the edge of each fungal isolate the mycelial plugs (5 mm in diameter) were excised and transferred to the center of appropriate Petri dish with 2% water agar plates and incubated at 28 °C during the seven days. After that, 1 mL of nematode suspension (containing 1000 nematodes) was added by the uniformly distribution of it on surface of each of plates for ensure the contact with testing fungi. As a control a nematode-free 2% water agar plate was employed. The predation structures of the studied fungi were observed at 24 h and 48 h time-intervals by stereomicroscopy method. Also, the quantity of predated nematodes was recorded [34].

Statistical assessment: All experiments were performed in triplicate wells and repeated three times independently under similar conditions. For the statistical assessment MS Excel program package was used [35,36]. For the digitization of images of microscopy ImageJ program packages were applied [37].

RESULTS and DISCUSSION

Samples of soil from agroclimatic different regions of RA were studied (table 1, Fig. 1).

Table 1. The differences in carnivorous fungi species diversity, isolated from the various agroclimatic zones of RA.

Agroclimatic zone	Isolated cultures	Occurrence frequency
Dry Tropical	5	0.11
Dry Sharp Continental	4	0.15
Dry Continental	4	0.11
Moderate Hot Dry	8	0.26
Moderate-warm Humid	13	0.51
Moderately Cold Humid	10	0.32
Moderate Cold	4	0.08
Cold	2	0.05

According to the obtained data, the fungal species diversity was different depending on the climatic zones of their isolation.The maximal quantity of isolated fungal species was found in moderate-warm humid agroclimatic zones, suggesting that the frequency of nematophagous fungi increased in humid zones of RA (Table 1). Also, the diversity found in moderate-warm humidzones contributed to larger quantities of strains of nematophagous fungi (Fig. 1).

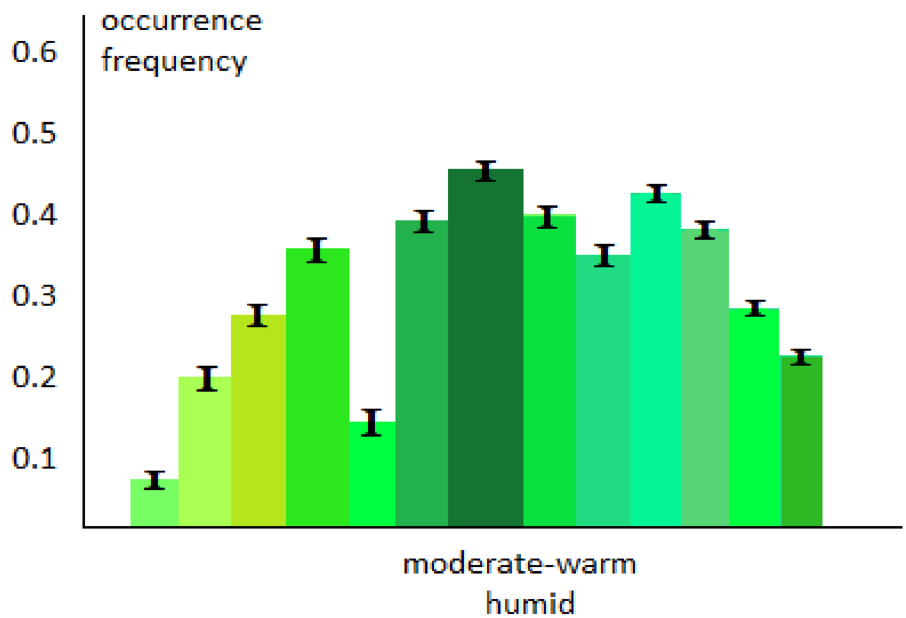


Fig. 1. Diversity of carnivorous (nematophagous) fungi, isolated from the moderate-warm humid agroclimatic zone of RA.

As a result of experiments, the following species were identified in different regions of RA: *Arthrobotrys conoides* Drechsler*, Golovinia appendiculata* Mekht.*, Drechslerella dactyloides* (Drechsler) M. Scholler (also known as *Dactylariopsis dactyloides* (Drechsler) Mekht.), *Nematoctonus haptocladus* (Drechsler) Mekht.*, Orbilia brochopaga* (Drechsler) Baral (also known as *Dactylariopsis brochopaga* (Drechsler) Mekht.). Then, their predation activity was studied by the stereomicroscopy (Fig. 2-4). The results of stereomicroscopy have demonstrated the presence of active traps and other predation structures in observed fungi.

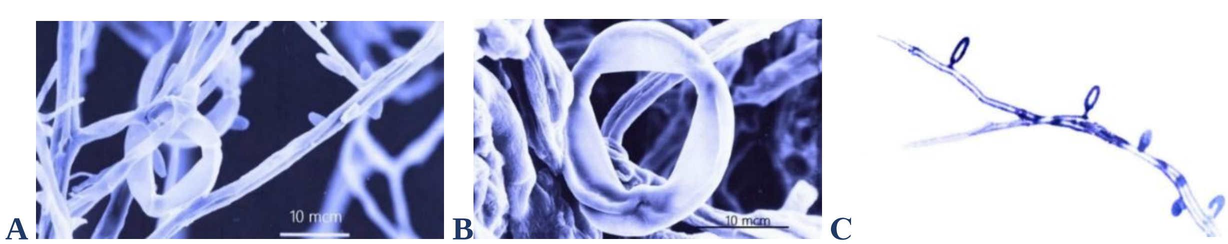


Fig. 2. Stereomicroscopy of trap-rings of (A) *Drechslerella dactyloides* (magnification x2000); (B) *Dactylariopsis brochopaga* (magnification x3200) and (C) the conidia of *Nematoctonus haptocladus* Drechsler (magnification x 500).

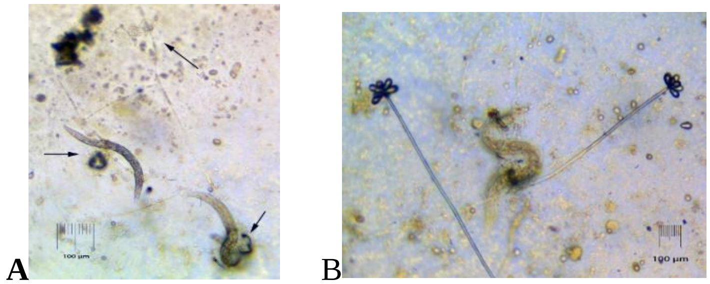


Fig 3. *Globodera rostochiensis* predation by *Orbilia oligospora*: immobilization of nematode in trap-ring (A), nematode body consumption by the mycelial hyphae penetration in worm tissues (B).

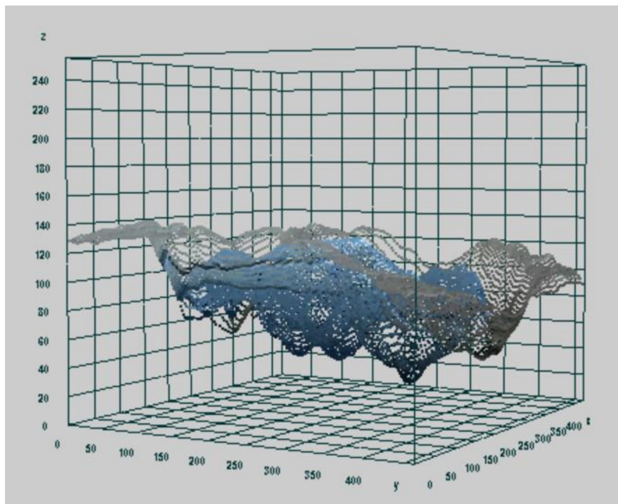


Fig. 4. Colorized digitization of predation activity comparison of different fungal isolates against the *Globodera rostochiensis*. Gray line: control samples; blue lines: the predation (nematicide) activity.

The results demonstrated that the RA predatory fungi occurrence frequency varied with different climatic zones, with maximal quantity of observed strains in warm humid zones. The differences in quantities of fungi for each sample may contribute to differences in the fungi's predatory activity. The maximal predatory activity was observed in case of fungi and nematodes, isolated in same region. In addition, all the strains of the isolated fungi have demonstrated the emphasized predatory activity against the potato hookworm. This result might potentially be used for further research of potato, tomato and other crops and plant protection, based on the fungi strains with the maximal predatory activity. The problem of contamination of potato and other vegetables by phytopathogenic nematodes, insects and other pests and their active secondary metabolites have important implications on healthcare and agriculture. The contamination of vegetables by nematodes and other pests, such as insect larvae (which have the worm-like phenotype), also has an ethical problem. For the religious customers from Muslim countries, the consumption of insects, worms, and similar products is traditionally prohibited [39]. In these regards, the elaboration of effective methods of vegetable protection is important, especially in terms of globalization [40-41].

The series of experiments, which were conducted have shown the presence of various predatory (carnivorous) fungi species in the soils of

Armenia. Nematicide activity was identified for all the isolated carnivorous fungi. During the experiments, the maximal nematicide effect was demonstrated in the application of local nematophagous fungi against the local phytopathogenic worms. This result may be related to the specificity of the enzymes that play a key role in important phases of infection of host-worm and the consumption of its body by the mycelial hyphae. In addition, the comparison of effects of the isolated cultures of nematophagous fungi and the commercially used nematophagous fungi-based preparations, based on foreign strains of fungi have demonstrated the higher effect of Armenian fungi: *Arthrobotrys conoides, Nematoctonus haplocladus, Golovinia appendiculata, Orbilia brochopaga.* .

Additionally, it should be noted that the warm and humid climate has a positive effect on carnivorous fungi diversity. Thus, the efficacy of the application of predation activity of nematophagous fungi against the phytopathogenic worms and the insect larvae might not be negatively affected with increases in global warming and climate changes.

In terms of fast development of green agriculture, as one of the most desired directions of sustainable economics, the innovation of pesticide-free applications, based on predation activity of fungi, may be very useful for RA. The application of agricultural preparations, based on nematophagous fungi as the technology of sustainable and green agriculture is the prospective alternative to classical pesticides. Carnivorous nematophagous fungi are safe for both human and mammalian animals [42]. Also, predatory fungi are able to synthesize various biologically active compounds with multiple potential of implementation possibilities [43-45].

The potential of nematophagous fungi application in agriculture and horticulture is very high. It offers the innovative approaches to solving the problem of pesticide-free agricultural products obtaining, with simultaneous significant decrease of contamination risks by both nematodes and their secondary metabolites. Deep and detailed research of carnivorous fungi might open the new possibilities for the obtaining of innovative antimicrobials, based on fungal secondary metabolites.

Due to the high resistance of fungal spores, the production and the storage of fungi-based biopreparations might have fewer financial expenses, in comparison to classical pesticides. Thus, this innovation application is beneficial for the economy [46-48].

The substitution of natural and artificial chemical preparations with appropriate fungal preparations, based on the species which are being isolated from, can significantly reduce the risks of environmental pollution while increasing the economic efficiency of agriculture. The agricultural application of the nematocidal preparations, based on individual fungal species or on the combination of various predatory fungi can potentially reduce the ecological risks for both soil and water ecosystems of active agricultural regions [49-51]. In these regards, the application of nematophagous fungal preparations in agriculture may significantly increase the efficiency and the safety of agriculture in farms which are situated near the lake Sevan.

In conclusion, the possibility of using carnivorous fungi in agriculture might be very prospective to increase tje quality of vegetables and crops. It offers novel prospectives on pesticide-free green agriculture technologydevelopment and minimization of risks from primary and secondary contamination of food products by harmful secondary metabolites of pathogenic nematodes, microbes and other pests. The application of nematophagous fungi in agriculture can bring a potential decrease of amounts of chemical contamination of water resources and soils, what is critical in XXI century. In addition, some secondary metabolites of nematophagous fungi might have antimicrobial effects which can offer an additional protective impact on the cultivating crops, and vegetables. Thus, the application of carnivorous fungi in agriculture can bring a huge progress in the industry of healthy and functional foods production.

Abbreviations: GMO, Genetically Modified Organisms; NTF, Nematode-trapping Fungi; Potato Dextrose Agar, PDA; RA, Republic of Armenia.

Author's Contributions: All authors contributed to this study.

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