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General characteristics of the means obtained from species belonging to the genus Trichoderma Karst distributed in Azerbaijan

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Abstract: In the conducted studies, 72 species belonging to sac fungi were isolated from the soil used for the cultivation of fruits and berries in pure culture. It became clear that among the recorded fungi, there are species that have antagonistic relationships with each other. Among these species, fungi belonging to the genus Trichoderma were observed with higher activity, with the highest activity recorded in species identified as Trichoderma asperellum and T. harzianum using classical methods. After identification through molecular techniques, it was found that the cultural solution obtained from the 5-day cultivation of the T. harzianum AEF-2024 strain on solid nutrient medium was suitable for research purposes. When diluted 50 times, this solution had the maximum effect on increasing the germination rate of various plant seeds. This finding opens wide prospects for using it to enhance the productivity of various plants (such as peas, tomatoes, wheat, etc.), especially those propagated by seeds.

Keywords: *Antagonism, Berries, Biostimulation, Culture solution, Fruits, Fungal biota, Soil.*

1. Introduction

Although the soil is characterized as a whole system that interacts with plants, it is also a natural environment where microorganisms are widespread, nourished, and participate in its formation. Its composition consists of both organic and inorganic components, which are formed as a result of the breakdown of materials characteristic of living organisms and the mineralization of organic substances. The content of these substances and the ratio of components depend on various factors, such as soil and climate conditions. For this reason, in order to optimize conditions for plant development and increase productivity, air, light, and temperature are also required, along with the soil [1]. Additionally, it is important for the life activities of plants and soil microorganisms to have a constant supply of new organic matter. This enables a deeper understanding of the multifunctionality of soils and the importance of the food chain that occurs there. It is necessary to note that biodiversity in the lower layer of the soil is much richer than in its upper layer, and this has not been comprehensively studied. Furthermore, the number of soil biota in 10 g of soil is 1.5 times greater than the number of people living on Earth [2].

It is known that the microbiota of soils is characterized by an extremely large number and includes mainly bacteria, fungi, protozoa, and others [3]. The primary role of these organisms is to participate in the process of metabolism and the self-cleaning of the soil that occurs in nature. As a result of their life activities, the destruction and mineralization of organic substances occur, enriching soils with new

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substances, regulating biodiversity, and other processes. More precisely, it is in their field that the substances that constitute the basis of life on Earth circulate. On the other hand, depending on the nature of the interaction of soil microorganisms, the natural climate-soil conditions of the environment, the intensity of microbiological processes, and the diversity of flora and fauna, the formation of productivity in areas suitable for agricultural purposes is determined. Behind this, the role of soil biota is extremely important. Thus, the biological activity of soils, as well as the number and activity of soil microorganisms, is determined by the amount and composition of organic matter in the soil [4].

Determining the indicators of biological activity of soils and using them to restore soil fertility, improve the phytosanitary condition, and increase the productivity of plants grown there is one of the urgent problems of the modern era [5]. Therefore, the fact that soil is one of the main areas where negative effects are observed as a result of increased human intervention in the environment in recent times is a reality that has long been accepted. This, in turn, suggests that using ecologically based methods and approaches to land use is an important issue. More specifically, it is also relevant in terms of realizing the use of nature and its resources by people in accordance with the principles of sustainable development.

According to the above, it can be noted that both the industrial and agricultural sectors have developed in the economy of the Republic of Azerbaijan, and both of these sectors have an impact on the environment [6]. The fact that Azerbaijan is a traditional oil country, as well as having one of the developed branches of the chemical industry, makes it inevitable that the environment will be polluted by raw materials and finished products from various production areas. This leads to a decrease in the area of land in the country, as well as a decrease in the fertility of the useful land and the productivity of the plants grown there [7, 8]. For this reason, it is necessary to improve the health of our land, protect it, increase the efficiency of its use, maintain its fertility, and produce the appropriate amount of products to meet the population's demand. In this direction, research conducted in various scientific centers around the world has determined that using the biological indicators of the soil, including microorganisms [6, 9] is beneficial for both ecological and economic considerations.

Microorganisms, including fungi, are widespread in various biotopes of the Republic of Azerbaijan [10] and they have been studied in various aspects, including as producers of biologically active substances [11]. However, the results obtained have not been widely used in practice. Therefore, the purpose of the presented work is dedicated to researching the possibilities of using fungi to increase the fertility of the soil used for the cultivation of various plants, primarily fruits and berries, and to increase the productivity of the plants cultivated there.

2. Material and Methods

Researches were conducted in the Guba-Khachmaz Economic District of the Republic of Azerbaijan. Samples for the study were taken from the soil where fruits were grown and analyzed for fungal biota. The process of taking soil samples, separating fungal cultures from them, determining their purity, and determining the antagonism and antibiotic activity of the isolated strains were performed according to the known methods [12, 13] used in the works of various authors. Both classical [14-17] and modern [18] mycological methods were used in the identification of fungi extracted for pure culture. According to the modern molecular-genetic method, identification was only carried out for two species belonging to the Trichoderma genus, which were selected as active producers as a result of screening.

Identification of Trichoderma fungi was carried out by the method of determining the direct nucleotide sequence of the ITS fragment, followed by determination of nucleotide identity with sequences deposited in the international Gene Bank [19] database, as well as by constructing phylogenetic trees with nucleotide sequences of reference strains [18, 20].

During the study of the antagonistic activity of the fungal species, which were determined to be clean and identified according to classical methods, the Canson-Karlo scale version improved by Alimova [21] was used. At this time, the assessment used in known methods was slightly modified and carried out according to the following:

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A=1 point – It is possible to cultivate the tested cultures together, that is, there was no antagonistic relationship.

B=2 points – The development of the two tested cultures occurs as soon as they come into contact with each other, and after a certain time, one of the cultures begins to develop weakly over the other.

C=3 points – Stoppage of development of both fungi after contact of cultures.

D=4 points – One of the cultures develops after contact with the other.

E=5 points – One of the tested cultures completely stops the development of the other, but at the same time, it continues its development by moving to the surface of its colony.

Finding the optimal conditions for the formation of maximum biomass during the cultivation of the selected cultures in liquid phase fermentation conditions was carried out in Czapek medium, which had the following (g/l): glucose (sucrose) – 30(20); yeast extract – 5; peptone – 5; KNO_3 – 2.5; KH_2PO_4 – 1; $MgSO_4 \cdot 7H_2O$ – 0.5; NaCl – 10; and agar-agar – 20. Sterilization conditions: 0.5 atm, 0.5 hours.

During the determination of the phytotoxic activity of the biomass and the culture medium (CM) produced by the fungi in the liquid nutrient medium, the CM was used directly or diluted. After washing the biomass produced by the fungi 3 times in phosphate buffer (pH = 7), 50 ml of sterile distilled water was added to it and passed through a tissue shredder (3 times for 3 minutes each). The resulting mixture was centrifuged (10 min, 5000 cycles), and the resulting solution (BE) was used. For phytotoxic activity, fungi were evaluated for their effect on the germination capacity of some plant seeds for both mentioned cases, which was carried out according to the methods used in our previous work [11].

During sampling and analysis, repeatability was at least 4 times, and all quantitative data were statistically processed [22]. In all cases, the results corresponding to the formula $\sigma / M = P \leq 0.05$ (where M is the average value of repetitions, σ is the standard deviation, and P is the Student's criterion) were considered valid and included in the dissertation.

3. Results and Analysis

The initial stage of the evaluation of soils according to mycological indicators involved the separation of pure cultures from the samples taken from this or other cenosic soils and the determination of their species affiliation. In this regard, about 100 samples taken from fruits *(Cydonia oblonga Mill., Prunus avium L., Malus domestica Borkh, Prunus persica Mill., Prunus dulcis Mill., Punica granatum L., Pyrus caucasica Fed. Ets.)*, *berries (Fragaria vesca L., Rubus idaeus L., Rubus alleghaniensis* Porter, etc.), and soil (used for cultivation of fruits and berries) in the study area were cultured. From these samples, 216 strains of sack fungi (*Ascomycota*) were isolated, and their species composition was determined by classical methods. Among the recorded genera, the largest species composition were the fungi belonging to the genera *Aspergillus (A. amstelodami, A. flavus, A. fumigatus, A. glaucus, A. nidulans, A. niger, A. ochraceus*, and *A. versicolor)*, *Fusarium (Fusarium avenaceum, F. culmorum, F. dimerum, F. gibosum, F. graminearum, F. moniliforme, F. solani*, and *F. verticillioides)*, *Penicillium (P. chrysogenum, P. cucullatum, P. expansum, P. divergensum, P. glaucium, P. jantinellum, P. lanosum, P. notatum, P. olivaceum, P. rubrum*, and *P. tardum)*, *and Trichoderma (T. atroviride, T. asperellum, T. citroviride, T. hamatum, T. harzianum, T. koningii, T. longibrachiatum*, and *T. viride)*. The number of species in these genera varied from 8 to 11. The number of species belonging to the remaining genera, such as *Alternaria (A. alternata, A. mali, A. solani, and A. tenuissima)*, *Ascochyta (Asc. berberides, Asc. fagi, and Asc. oleae)*, *Botrytis (B. cinerea)*, *Cercospora (C. beticola* and *C. flagellaris)*, *Cladosporium (C. cladosporioides and C. herbarum)*, *Cylindrocarpon (C. album)*, *Cytospora (C. amygdali and C. punicae)*, *Gliocladium (G. hirsutum and G. roseum)*, *Gloeosporium (G. fructigenum and G. coryli)*, *Monilia (M. fructigena and M. laxa)*, *Nectria (N. cinnabarina)*, *Phyllosticta (Ph. elagni, Ph. oleae*, and *Ph. punicae)*, *Polystigma (P. amygdalinum* and *P. fagicola)*, *Septoria (S. amygdali, S. oleae, S. pistacina, S. pruni*, and *S. rubi)*, *Stigmatea (S. aegopodi)*, *Venturia (V. inaequalis* and *V. oleaginea)*, *and Verticillium (V. albo-atrum* and *V. dahliae*), varies from 1 to 5. Most of the 72 recorded fungal species were

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phytopathogenic to one degree or another, and this has been confirmed both by literature data and by our observations. Nevertheless, they differ from each other in terms of the quantitative indicator of phytopathogenicity, that is, the degree of damage caused by the diseases they cause to the productivity of the host plant, their toxicity, and other characteristics. Endophytes were also found among the recorded fungi. From this point of view, the recorded diversity plays a certain role in the manifestation of this or that feature of a specific fungus, the abiotic and biotic environment, including the relationships between the fungi. Clarification of this is important for the prevention of negative and positive processes occurring in soils, primarily those used for the cultivation of plants of various purposes. This has been confirmed in a number of studies [7, 21, 23].

Taking this into account, the antagonism feature of the registered fungi was studied, and the fungi that have an antagonistic relationship with phytopathogens were specified. From the obtained results, it became clear that antagonistic relationships between fungi are observed to a certain extent, and this is most evident in fungi belonging to the genus *Trichoderma*. Thus, species such as *T. atroviride*, *T. asperellum*, *T. citroviride*, *T. hamatum*, *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *and T. viride* of the genus *Trichoderma*, recorded in the studies, exhibit antagonism in relation to fungi such as *Alternaria alternata*, *A. mali*, *A. solani*, *Ascochyta berberides*, *Botrytis cinerea*, *Fusarium avenaceum*, *F. culmorum*, *Monilia fructigena*, *Trichothecium roseum*, *Venturia inaequalis*, *Verticillium albo-atrum*, *V. dahliae*, and others, which are considered strong phytopathogens, and its quantitative indicator was between 3 and 5 on a 5-point scale. Antagonism between other species was expressed by 1-2, and in rare cases (species belonging to the genus *Gliocladium*) by 3. As a result of the research conducted at this stage, 2 species with the highest antagonistic activity (*T. asperellum* and *T. harzianum*) were selected for the next stage of research, and first, their species composition was clarified by molecular-genetic methods. Although one of these fungi was confirmed to be T. harzianum according to the nucleotide sequence, the fungus identified by the classical method as T. asperellum was determined to be a mixture of taxa such as T. citrinoviride and Trichoderma harzianum. Due to the difficulty in determining the specific characteristics of the mixed fungus, only *T. harzianum* was used in the studies. The most active strain specific to that species was named as *T. harzianum* AEF-2024, which was determined to be 99.66% identical to the strain with inventory number MH865865.1 in GeneBank [23] according to the nucleotide sequence of the ITS gene (Table 1)

Table 1.
Nucleotide sequence of the ITS fragment of the fungus *T. harzianum* AEF-2024.

TTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCAATGTGAACGT
TACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGG
CGCCCGCCGGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTTTTTTTATAA
TCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC
GGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGG
GGATCGGCCCTCCCTtAGCGGGGTGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAG
CCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTA
AACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCT

As a conclusion of the studies, the fungus was cultivated in the environment optimized in our previous works to determine the effects and areas of use of the metabolites synthesized by the T. harzianum AEF-2024 strain selected as an active producer. The obtained biomass was filtered, and both the obtained biomass and the culture medium were used as the target product. Although the culture solution (CS) was used directly or by diluting with water, the biomass was passed through a tissue grinder in sterile distilled water in specific amounts, and the obtained solution (biomass extract - BE) was used. The obtained products were first investigated for their effect on plant seed germination, that

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is, for phytotoxic activity. Phytotoxic activity was not observed either in the biomass or in the culture solution obtained after 5 days of cultivation of the fungus in Czapek medium. On the contrary, stimulation was observed in all the tested plant seeds (Table 2). As can be seen, in all cases, the stimulation phenomenon observed during the use of CS was higher than that of BE, which allows us to note that the metabolites causing stimulation were exogenous and water-soluble.

Table 2.
The effect of the preparations obtained from the fungus *T.harzianum* AEF-2024 on the germination capacity of plant seeds(according to the number of seeds that did not germinate %).

Tested means	Dilution, times	Wheat	Peas
Biomass extract	7.5	9.5	8.0
Culture solution	5.0	6.5	5.5
Control	8.5	10.5	9.5

From the studies conducted to determine the stimulation-causing concentration of the obtained means (BE and CS), more precisely the effectiveness of their direct or diluted use, became clear that dilution of BM by 25 times and CS by 50 times results in the stimulation event being characterized by comparatively higher rates Table 3.

Table 3.
The effect of changing the concentration of the preparations obtained from the fungus *T.harzianum* AEF-2024 on the germination capacity of plant seeds (in % relative to the initial).

Tested means	Dilution, times	Wheat	Peas	Tomato
Biomass extract	0	100	100	100
	10	102	103	104
	25	107	106	105
	50	106	106	105
	75	105	104	104
	100	103	102	102
Culture solution	0	100	100	100
	10	102	103	104
	25	106	107	108
	50	111	110	112
	75	109	108	110
	100	107	106	108

These results also confirm the promising use of *T. harzianum* AEF-2024 as a biostimulant during the cultivation of various plants, especially those grown from seeds. This will also be clarified in future studies. One of the points that needs to be clarified in the future is the determination of the possibilities of using the agent (CS) obtained from the fungus *T. harzianum* AEF-2024 in increasing the productivity of vegetatively propagated fruit and berry plants. Some berry plants reproduce vegetatively (using cuttings and young shoots), while others reproduce by seed, more precisely, they reproduce sexually and asexually. Asexual reproduction, including vegetative reproduction, is considered more favorable due to biotechnological considerations [24].

The increase in the productivity of seed-propagated plants as a result of the influence of various bioagents, including those derived from fungi [4, 25] has been confirmed in various studies, including ours [26]. However, this is mainly related to vegetable crops cultivated in agriculture. For this reason, the issue of increasing the productivity of asexually reproducing plants should be the focus of researchers in the future. This is also important for the efficient use of resources and their management in accordance with the principles of sustainable development.

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4. Conclusions

From the research carried out, it became clear that the soil used for the cultivation of fruits and berries is one of the habitats of fungi, and the species belonging to the sac fungi distributed there were characterized by a wide diversity due to the manifestations of ecotrophic specialization. Determining the relationship (antagonism) of these fungi to each other was important from the point of view of their effective use. The determination of the antagonism of the strains belonging to 72 species of fungi isolated during the research confirmed that fungi with this feature were present among them, and some exhibited maximal antagonism according to the existing methodological approach. As an active producer, it was convenient to use the culture solution obtained from T. harzianum AEF-2024 as a biostimulant to increase the productivity of plants cultivated from seeds in a liquid nutrient medium. This, in turn, stimulates the expansion of research aimed at finding means to increase the productivity of plants, including fruits and berries, during vegetative propagation rather than by seed. In addition, fungi identified by classical mycological methods are not always pure, and for these purposes, the molecular-genetic approach can be considered non-alternative.

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Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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