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ADVANTAGES AND DISADVANTAGES

of two modications of the biological method of analysis of wheat seed mycobiota

T. ROZHKOVA,
PhD in Biology
Sumy National Agrarian University,
160, G. Kondratieva str., Sumy,
40021, Ukraine
e-mail: rozhkova8@gmail.com

Goal. *To determine the most effective modification of the biological method of analysis of mycobiota of winter wheat seeds.* ***Methods.*** *Laboratory analysis of mycobiota of winter wheat seeds by biological method on PGA and on filter paper (wet chamber, rolls), determination of fungi on PGA medium on the basis of modern revision of taxa; analytical and mathematical — analysis of the obtained results and their statistical comparison.* ***Results.*** *During the first phytoexpertise of seeds in 2007, a significant percentage of fungal infections was 37.6%, which raised doubts and led to the next area of research — the comparison of modifications of the biological method. In 2008, phytoexpertise of wheat seeds of four varieties (Driada, Podolyanka, Odeska 267, and Pysanka) was carried out on PGA and on paper rolls. Statistical comparison of the results of fungi of all seeds, determined by the two modifications, was insignificant. In 2010, the analysis of seeds on three varieties (Ukrayinka poltavs'ka, Odes'ka 267, and Dons'ka) showed a significant difference between the results obtained on different substrates. More colonies were isolated on the PGA than on paper rolls. Alternaria and Fusarium fungi were isolated more on agar medium than on paper rolls when comparing the characteristics of infection by individual genera. In 2020, we compared the effectiveness of the analysis of seed mycobiota on agar and paper on the variety of Bohdana from the Forest-Steppe and Polissya, finding more isolation of fungal colonies and a wider range of fungi on the PGA.* ***Conclusions.*** *Phytoexpertise of wheat seeds in 2010 showed a significant difference between the amount of total infected seeds and separately seeds with Fusarium and Alternaria fungi on PGA and paper rolls. The analysis of the mycocomplex of seeds at the PDA identified a new direction of research: from the detection of seed contamination to the settlement of fungi, and then — to the analysis of mycobota with the determination of the percentage of genera / species among the total amount of fungi. Analysis of the micocomplex in 2020 on agar and in a wet chamber showed best results of the first modification of the biological method. But it has disadvantages: the growth of polluting fungi, parasitizing mycophilous fungi. Analysis of mycobiota on filter paper has a rapid demonstration result, but does not show the full range of fungi. Therefore, it is better to use agar media for research.*

biological method; PGA; wet chamber; paper rolls; mycobiota analysis; seed; winter wheat

The seed contains various microorganisms inside, which today are considered as the microbiome. The majority of this microbial complex is made up of fungi, the role of which is still not fully understood. It is known that some of them are phytopathogenic species that persist on or inside seeds, causing infection at the first stages of plant development, especially during germination. But most fungi are endophytes, which do not have a negative effect on plants, but have a positive effect on plants, producing secondary metabolites, increasing drought tolerance and resistance to pathogens, stimulating plant growth and development. The endophytic microbiota is associated with the host plant throughout its ontogenesis [1—3].

In the world, for the most part, not the entire spectrum of wheat seed fungi is studied, but more common or harmful ones. Fusarium sp., Penicillium sp., Aspergillus sp. are the most harmful species due to their ability to produce mycotoxins. Especially dangerous are *Fusarium* fungi with the widest range of secondary metabolites, which are constantly being investigated for the reasons of their significant harm to plants, as well as humans and animals. They cause significant economic losses, reducing the quantity and quality of products due to their contamination with mycotoxins [4]. *Alternaria* fungi are the most common in the mycocomplex of wheat seeds. In recent years, *Alternaria* sp. have a significant percentage of isolation among seed fungi in the world: in Ukraine [5, 6], in the countries of the European Union [7, 8], in the north of Africa [9], in South America [10, 11], Russia [12] and countries of Asia [13, 14].

There are fewer studies on the determination of mycobiota of wheat seeds due to the difficulty of diagnosing all species. According to the analysis of the literature, we established that in the mycobiota of wheat seeds in the 21st century the following genera were identified in the world: *Acremonium* sp. *Alternaria* sp. (*Ulocladium* sp. belong to *Alternaria* sp.), *Arthrinium* sp., *Aspergillus* sp., *Aureobasidium* sp., *Cephalosprium* sp., *Chaetomium* sp., *Cladosporium* sp., *Cochliobolus* sp., *Curvularia* sp., *Epicoccum* sp., *Fusarium* sp., *Gliocladium* sp., *Microdochium* sp., Mortierella sp., Mucor sp., Nigrospora sp., Penicillium sp., Phoma sp., Pyrenophora sp., *Rhizo-*

pus sp., *Rhizoctonia* sp., *Stemphylium* sp., *Trichoderma* sp., *Trichothecium* sp. and *Verticillium* sp.

Currently, biological and molecular methods are used to analyze the fungal seed complex. The biological method is more accessible to Ukrainian scientists, so most of the results were obtained using it. Only *Fusarium* fungi from wheat seeds in Ukraine are diagnosed using PCR analysis [15]. The biological method has two modifications: seed germination in a moist chamber and on an agar medium. A scientist who starts research always faces the question of choosing a modification. Therefore, we compared them to identify the wheat seed mycobiota most suitable for analysis.

The purpose of the research is to determine the most effective modification of the biological method of analysis of mycobiota of winter wheat seeds.

Materials and methods. Seed samples were obtained from various farms of the Sumy region. Bohdana variety was grown in the conditions of the Forest Steppe (Educational and Scientific Production Complex of the Sumy National Agrarian University) and in the conditions of Polissia (Shostka district). Analysis of seed mycobiota was carried out by biological method in two modifications according to DSTU 4138:2002. Seeds were germinated on filter paper (rolls and circles in Petri dishes) and on potato-glucose agar medium. Before decomposition, the seeds were washed under a stream of cold water, kept in a 1% solution of potassium permanganate for 1—2 minutes and washed with sterilized water. Dishes and rolls were incubated in a thermostat at a temperature of 22—24°C for seven days. Fungi were identified according to T. Yu. Gagkaeva et al. [16], T. Watanabe [17], K. Schubert et al. [18], P. Zalar et al. [19], G. Walther G. [20] and E. J. Warham et al. [21].

Results and discussion. Scientific research was started with phytoexpertise of winter wheat seeds according to the current standard for determining seed infection, stopping only on work with phytopathogenic species. It was conducted for the first time in 2007 at the PGA, obtaining significant indicators of seeds colonized by fungi — 37.6%, as we believed at that time. We have doubts about the chosen method. The biological method, in our opinion, allowed the most effective isolation of phytopathogenic species. It is the most common for determining fungal infection of cereal seeds, and allows detecting internal infection. This method is based on stimulation of the development of microorganisms, allows establishing the type of pathogen and the degree of seed infection [22]. Having experience in working with nutrient media and wet chambers, we understood the peculiarities of their implementation and effectiveness. Therefore, we faced the task of determining the most effective modification of the biological method.

First, phytoexpertise of wheat seeds from four varieties (Driada, Podolyanka, Odeska 267, Pysanka) of the 2008 harvest was carried out with two modifications: on PGA and paper rolls. Various results were obtained on the damage by fungi of individual varieties. But counting the total number of seeds with fungal colonies and their statistical comparison showed no significant difference between the two modifications. It was possible to draw conclusions on the isolation of *Fusarium* and *Alternaria* fungi: the former were better isolated on a nutrient medium, the latter on paper rolls [23]. In 2010, phytoexpertise of wheat seeds in these two modifications was conducted on three varieties (Ukrayinka poltavska, Odeska 267, Donska). This time, a significant difference was noted between the results obtained on different substrates (Fig. 3.1).

According to the results of 2010, it was possible to draw conclusions about the greater effectiveness of the phytoexpertise conducted with the help of a nutrient medium — KGA, when 85% of the seeds with fungal colonies were detected.

In addition to the number of seeds with mushrooms, it was determined that they were species of *Fusarium* and *Alternaria* fungi. The study of the presence of colonies of different species inside the seeds also showed higher indicators on the nutrient medium (Fig. 2).

More *Alternaria* and *Fusarium* fungi were isolated on the PGA medium than on paper rolls, which was confirmed by their statistical comparison: $LSD_{05}A=15.4$, $LSD_{05}F=3.7$.

They were able to compare the results of the phytoexpertise of the harvests of two years due to the analysis of one Odeska 267 variety (Fig. 3).

In 2008, the difference in seeds with fungi between the two substrates was only 2%, and in 2010, it was 14.5% (and it turned out to be significant: $LSD_{05}=7.9$) in favor of the modification using the nutrient medium. A larger number of individual genera were also iden-

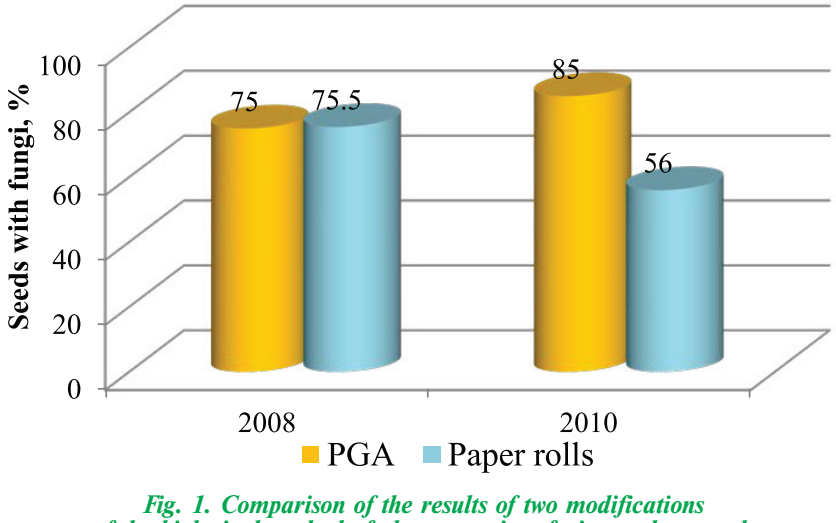

Fig. 1. Comparison of the results of two modifications of the biological method of phytoexpertise of winter wheat seeds (Sumy district, 2008, 2010) ($LSD_{05}10 = 7.3$)

tified on the PGA ($LSD_{05}F$=5.1; $LSD_{05}A$=4.8).

For the comparison of two methods of seed analysis (blotter method — filter paper, agar method) A. A. Mohmed et al. [24] determined a greater number of fungi of the genera *Aspergillus* and *Penicillum* on agar than on paper.

According to the results of two years of research, especially according to the data of the last year, we decided that for further phytoexpertise of winter wheat seeds, we should use a nutrient medium that allowed us to detect the largest number of seeds that contained fungi inside.

The modification of the biological method chosen by us with the use of a nutrient medium gradually changed the vector of our research. Other fungi, which we had no idea about after studying all the special literature on phytopathology, began to be actively isolated on the nutrient medium. Cases of the presence of several fungi inside one seed were found. We began to determine not the number of seeds with fungi, but the percentage of the release of fungi among all colonies. That is, we gradually switched from conducting phytoexpertise to studying the mycobiota of winter wheat seeds. Having many years of experience working with various components of the mycocomplex of seeds, we decided to once again compare in more detail different substrates for the isolation of fungal colonies. PGA medium and filter paper were also used, but this time its circles were placed in Petri dishes. The work on determining the number of fungal colonies turned out to be more effective compared to the previous determination of settled seeds (results of 2008 and 2010).

Counting the colonies that germinated on the nutrient medium and on the filter paper for the same number of seeds showed a greater number of fungi from the seeds than the first method (Table 1).

The difference in colonies was found from seeds grown under different conditions of crop cultivation. Almost twice as many fungal colonies sprouted on agar medium from seeds from the Forest Steppe than on filter paper. The number of colonies from seeds from Polyssia on different substrates differed less than in the Forest Steppe.

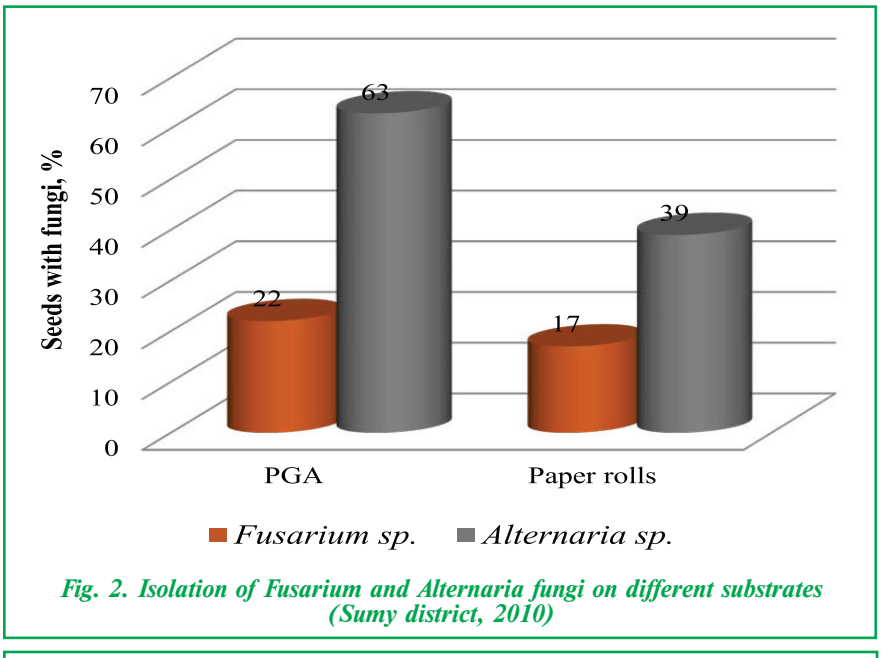

Fig. 2. Isolation of Fusarium and Alternaria fungi on different substrates (Sumy district, 2010)

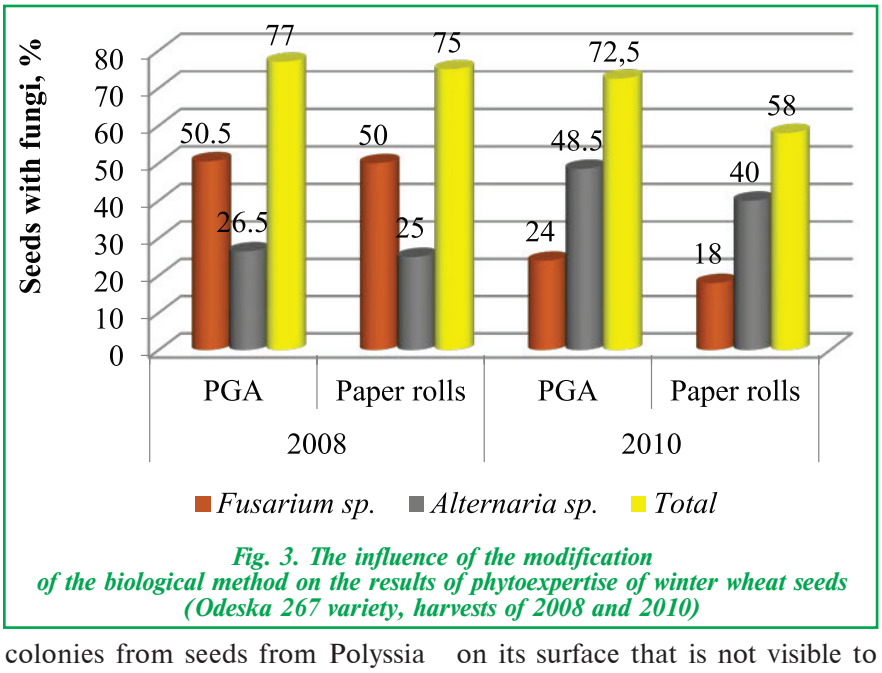

Fig. 3. The influence of the modification of the biological method on the results of phytoexpertise of winter wheat seeds (Odeska 267 variety, harvests of 2008 and 2010)

In addition, the visualization of the results is an important factor that affects the further decision regarding damage to a certain batch of seeds. Even a researcher may not pay attention to a seed that has germinated well, but has a coating on its surface that is not visible to the naked eye. He will pay more attention to seeds with abundant development of the fungus, missing the fact of seed damage. Microorganisms that form inconspicuous plaques, regardless of their area, will be ignored (Fig. 4).

From fig. 4, it is immediately visible that when using a wet chamber,

1. The number of fungal colonies based on the analysis of winter wheat seeds by two modifications of the biological method (Bohdana variety, 2020)

Growing area	The number of colonies per 100 seeds, pcs		LSD_{05}
	Agar medium	Filter paper	
Forest steppe	143	79	12.2
Polissya	105	71	11.3

almost half of the seeds do not have plaques. On the medium, we can clearly see the germination of dark colonies from each seed. Counting plaques showed a greater number of them on the seeds, but microscopy proved the presence of sporulation on the externally unaffected grain.

A detailed examination of each seed using a microscope showed quite interesting results, compared to the initial examination of the Petri dishes (Table 2).

Examination of fungal plaques with the help of a microscope from seeds spread on filter paper showed that they were formed by *Alternaria* fungi. A detailed study of the supposedly «pure» seed revealed the presence of sporulation, which consisted of chains of these hyphomycetes. Several deposits were also noted, which consisted exclusively of mycelium of mushrooms. With the help of PGA, it was possible to isolate a wider spectrum of fungi than with the help of filter paper. Simultaneous germination of several colonies from one seed was noted. Usually, an *Alternaria* fungus could germinate together with another. The number of fungal colonies was greater on the agar medium. The ratio of fungi in the seed mycoflora varied depending on the substrate for analysis, which was associated with a wider representativeness of fungi on agar.

The complex of fungi from seeds grown in the conditions of the Forest-Steppe was more diverse than in the Polissya zone (Table 3).

If in the Polissya zone the percentage of selection of dominant *Alternaria* fungi on different substrates did not differ so much, then the sample from the Forest Steppe showed a higher indicative capacity of the agar medium than the filter paper. It was possible to obtain almost twice as many colonies. On filter paper, out of 79 colonies, 60.8% were *Alternaria* sp., and on agar, out of 143 colonies, 58.8% turned out to be *Alternaria* fungi. A greater number of species and genera of fungi were also determined on agar. From one wheat seed grew from 1 to 3—4 fungal colonies. The simultaneous appearance of the *Alternaria* fungus, *T. roseum* and an unknown mycelium was most often observed. The ratio of fungi in the mycoflora of wheat seeds from the Forest-Steppe zone was also determined by the substrate for the isolation of fungi, but the proportion of *Alternaria* fungi was almost the same.

Fig. 4. Visualization of the results of the biological method (7th day) using different substrates (filter paper, PGA) (Bohdana variety, Polissya, 2020)

Conducting a comparison of the substrate for the detection of mycoflora fungi after many years of experience with working on the environment confirmed the correct choice of this modification at the beginning of the research. A wider spectrum of fungi is distinguished on the medium, they grow better, they can be easily taken for reseeding and further identification, and the better development of fungi allows you to follow the features of the development of plants in their significant presence. But over many years of research, there were certain difficulties with the growth of contaminating fungi (*Neurospora sitophila* Shear, *Mucor mucedo* L. та *Rhyzopus stolonifer* (Ehrenb.) Vuill.).

In India, when analyzing mycoflora on filter paper and on agar medium with pretreatment of wheat seeds with potassium nitrate, more

2. The influence of the substrate on the results of phytoexpertise of winter wheat seeds (Bohdana variety, Polissya, 2020 harvest)

Substrate	The percentage of fungal colonies isolated from their total number,%
Filter paper	*Alternaria* sp. — 94.4 Others species of fungi 5.6
Agar medium	*Alternaria* sp. — 73.3 *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud. — 21.0 *Cladosporium herbarum* (Pers.: Fr.) Link — 1.9 *Mucor mucedo* L. — 1.0 Others species of fungi — 2.8

3. The influence of the substrate on the results of phytoexpertise of winter wheat seeds (Bohdana vari-ety, Forest-Steppe, 2020 harvest)

Substrate	The percentage of fungal colonies isolated from their total number,%
Filter paper	*Alternaria* sp. — 60.8 *Penicillium* sp. — 10.6 *Rhyzopus stolonifer* (Ehrenb.) Vuill. — 8.4 *Trichothecium roseum* (Pers.) Link — 8.4 Others species of fungi — 11.8
Agar medium	*Alternaria* sp. — 58.8 *T. roseum* 17.5 *Trichoderma* sp. — 2.8 *Penicillium* sp. — 2.1 *Harzia acremonioides* Les Mucédinées — 2.1 *Fusarium sporotrichioides* Sherb. — 0.7 Others species of fungi — 13.9

fungal species were isolated by the first method, which was explained by the fact that the fungi were suppressed by other fast-growing ones, and the environment was unfavorable for the development of certain species [25].

According to our observations, the use of filter paper is appropriate for the study of *Alternaria* fungi. Working with them on a nutrient medium had a lot of problems for us due to their coexistence with other fungi, especially mycophilous ones. They often destroyed the characteristic habit of sporulation of *Alternaria* fungi, and it was also difficult to get rid of them when transplanting into a pure culture (Fig. 5).

A more detailed study of them on filter paper showed good sporulation growth, especially on the surface of the seed itself. That is, if the goal is only to identify species of the genus *Alternaria*, then an effective option is to grow them from seeds on paper with subsequent transplanting to special media to confirm the branching of sporulation. Ph.B. Hannibal recommended preliminary isolation of *Alternaria* fungi in a humid chamber [12].

Thus, we observed well-developed sporulation with typical habits characteristic of *A. alternata, A. tenussima,* and *A. arborescens* on the filter paper in the seed sample from the Forest Steppe. Fungal coexistence was often encountered on the nutrient medium when *Alternaria* sporulation was underdeveloped. From the seeds grown in the Forest-Steppe conditions, well-formed spores of *A. arborescens*, *A. avenicola*, and *A. alternata* were often found on filter paper (Fig. 6). At that time, the presence of only *A. arborescens* was noted on the nutrient medium.

The research was carried out within the framework of the budgetary scientific topic «Phytoexpertise of seed material and food grains of cereal grain crops» 0115U001875.

CONCLUSIONS

At the beginning of the research, based on the results of two years of analyzes (2008 and 2010), the most optimal modification of the phytoexperisis of wheat seeds using a nutrient medium (PGA) was determined, as it allowed to isolate the largest number of seeds with fungal colonies. Phytoexpertise of wheat seeds at PGA changed the research vector: from determination of seed contamination to population, and then to the analysis of seed mycobiota, i.e. determination of the presence of species among all fungal colonies. A comparison of different substrates for the analysis of the mycobiota of seeds from different agro-climatic zones (Forest Steppe and Polissia) in 2020 showed a lower number of fungal colonies on filter paper than on agar medium. But it is better to germinate *Alternaria* sp. on seeds on filter paper, as they germinate well on the surface of the seeds with subsequent transplanting to special media to confirm branching of sporulation. That is, the analysis of seeds on an agar medium allows to determine a wider species spectrum and to germinate a larger number of fungi, but it has disadvantages: the growth of contaminating fungi, parasitism of mycophilous fungi. Analysis of the mycobiota on filter paper has a quick indicative result, but does not demonstrate the entire spectrum of fungi. Therefore, it is better to use agar media for scientific research.

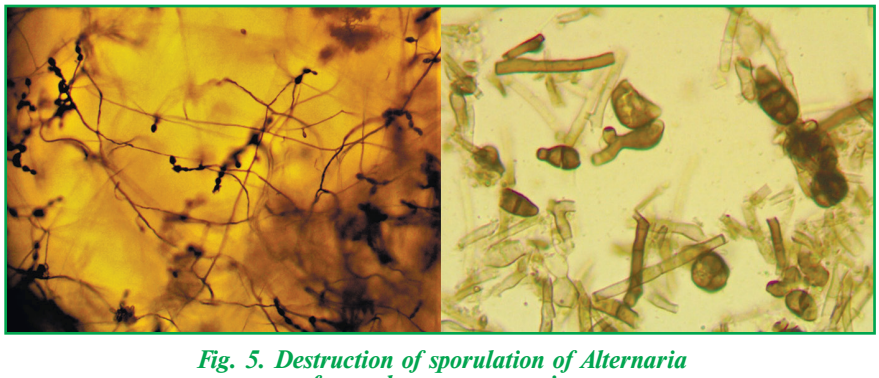

Fig. 5. Destruction of sporulation of Alternaria fungus by a mycoparasite

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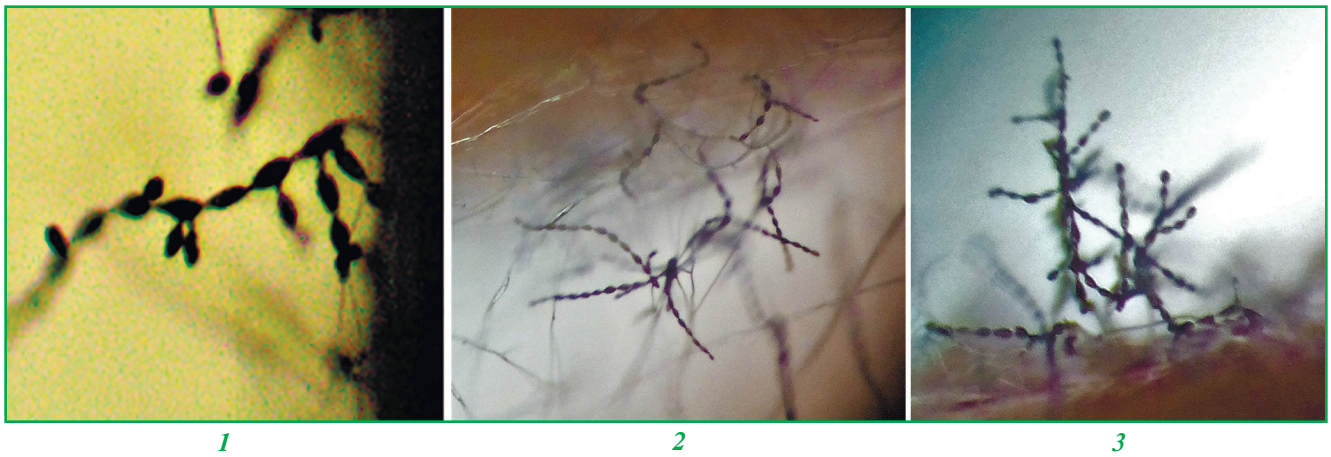

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Fig. 6. Sporulation of different species of Alternaria from the surface of seeds on filter paper (A. avenicola from Polissia, A. tenussima and A. arborescens from Forest-Steppe)

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Рожкова Т.О.
Сумський національний аграрний університет, вул. Г. Кондратьєва, 160, м. Суми, 40021, Україна
e-mail: rozhkova8@gmail.com

Переваги та недоліки двох модифікацій біологічного методу аналізу мікобіоти насіння пшениці

Мета. *Визначити найефективнішу модифікацію біологічного методу аналізу мікобіоти насіння пшениці озимої.* **Методи.** *Лабораторний — аналіз мікобіоти насіння пшениці озимої біологічним методом на КГА та на фільтрувальному папері (вологa камера, рулони), визначення грибів на середовищі КГА на основі сучасної ревізії таксонів; аналітичний та математичний — аналіз одержаних результатів та їх статистичне порівняння.* **Результати.** *За першої фітоекспертизи насіння у 2007 р. одержали значний відсоток зараження грибами (37,6%), що викликало сумніви та привело до наступного напряму досліджень — порівняння модифікацій біологічного методу. У 2008 р. фітоекспертизу насіння пшениці чотирьох сортів (Дріада, Подолянка, Одеська 267, Писанка) провели на КГА та на паперових рулонах. Статистичне порівняння результатів заселеності грибами всього насіння, визначеної двома модифікаціями грибів, було неістотним. У 2010 р. аналіз насіння трьох сортів (Українка полтавська, Одеська 267, Донська) показав істотну різницю між результатами на різних субстратах. На КГА виділили більшу кількість колоній, ніж на паперових рулонах. За порівняння особливостей зараження окремими родами зробили висновок, що альтернарієвих та фузарієвих грибів було виділено також більше на агарі. У 2020 р. порівняли результативність аналізу мікофлори насіння на агарі та папері на сорті Богдана з Лісостепу та Полісся і встановили більшу кількість виділення грибних колоній та ширший видовий спектр грибів — на КГА.* **Висновки.** *Фітоекспертиза насіння пшениці у 2010 р. продемонструвала істотну різницю між кількістю всього зараженого насіння та окремо з фузарієвими й альтернарієвими грибами на КГА та паперових рулонах. Аналіз мікокомплексу насіння на КГА визначив новий напрям досліджень: від виявлення зараженості насіння до заселення грибами, а потім — до аналізу мікобіоти з визначенням відсотка колоній родів/видів серед всієї кількості грибів. Аналіз мікокомплексу в 2020 р. на агарі та у вологій камері показав кращу результативність першої модифікації біологічного методу. Але вона має недоліки: розростання грибів-забруднювачів, паразитування мікофільних грибів. Аналіз мікобіоти на фільтрувальному папері має швидкий показовий результат, але не демонструє всього спектра грибів. Тому для наукових досліджень краще застосовувати агарові середовища.*

біологічний метод; КГА; волога камера; паперові рулони; аналіз мікобіоти; насіння; пшениця озима

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