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*ORIGINAL ARTICLE*

# Influence of seed treatment on microbiota and development of winter wheat seedlings

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The microbiota of winter wheat seeds from the North-East of Ukraine was studied by a biological method. Its considerable variability is established over three years (2017–2019). The effect of the treatment agents on most microorganisms of wheat seed microbiota in Ukraine, rather than on its genera and species, is shown. It has been proven that fungicides deleted some species and did not affect the development of others. Chemicals replaced some species or genera of fungi with others or even other microorganisms. Biological seed treatment (Phytosporin-M) has caused less microbiota change than chemical treatment (Maxim 0.25 FS, Rostock, Kinto Duo). Fungicides have replaced the dominance of *Alternaria* spp. (2017 – 57.8%, 2018 – 63.5%) for the dominance of yeast (Rostock – 54%) and *Aureobasidium pullulans* (Maxim 0.25 FS – 84.2%) in 2017, bacteria (Maxim 0.25 FS – 72.3%, Rostock – 53.8%) – in 2018. *A. pullulans* dominated in the microbiota of winter wheat seeds in 2019. The highest amount of *A. pullulans* was noted for the treatment of seeds by Phytosporin-M (85.9%). The biological seed treatment reduced the amount of *Nigrospora* spp. and *Alternaria* spp. Several times (3 and 5, respectively), chemical agents did not give *Nigrospora* spp. germination reduced the amount of *A. pullulans*, *Alternaria* spp. in 2019. Maxim 0.25 FS, Rostock 50%, and Kinto Duo delayed seed germination and seedling development on agar medium and soil, whereas Phytosporin-M – on the contrary, promoted the growth of seedlings and significantly exceeded control.

**Keywords:** seed microbiota, winter wheat, seed treatment, seed-borne fungi, fungicides

## Introduction

In Ukraine, wheat is the main food crop. The area under this crop equals $6 \cdot 10^6$ ha; the wheat yield is $2.5 \cdot 10^7$ t annually. The wheat yield quality and amount are limited by pathogenic microorganisms that form part of the plant microbiota.

Microbial communities of plants include bacteria, fungi, protists, and viruses. Microorganisms interact with plants in different ways: they are used as a source of nourishment, enter into symbiotic relationships, and inhibit the development of plant pathogens (Rodriguez et al., 2009). Many modern scientific works are aimed at the study of endophytes. They are the microorganisms that reside inside healthy plant tissues without causing any detectable disease symptoms to the host (Gond et al., 2010).

Seed-borne fungi are parasites of plants, nematodes, other fungi, symbionts, and endophytes. Pathogenic fungi of seeds are much broader studied. In the world, mycoflora of wheat seed consists of such genus as *Alternaria, Aspergillus, Ceratobasidium, Cercospora, Cochliobolus, Curvularia, Drechslera, Fusarium, Gaeumannomyces, Microdochium, Penicillium, Pyricularia, Pythium, Rhizoctonia, Rhizopus, Sclerophthora, Trichoderma,* and *Tricoconella* (Miller, 1995). Fungi of winter wheat mycoflora have not been studied enough. Seed-borne fungi are periodically studied in different countries. Thus, the mycoflora of wheat seeds (*Triticum aestivum*) in Nepal comprised 18 fungi species of 13 genera. The representativeness of fungus species was determined by the variety and the location of its cultivation. *Alternaria alternata* and *Bipolaris sorokiniana* dominated in all research variants (Adhikari et al., 2016; Adhikari et al., 2018). Twenty-one genera/species were found in Latvia during the period

of investigation 2012–2016. *Pyrenophora tritici-repentis*, *Alternaria* spp., *Arthrinium* spp. and *Fusarium avenaceum* were most widely spread and distributed in the mycoflora (Bankina et al., 2017).
The researchers paid much attention to the most pathogenic and widely spread mycoflora species of wheat seed, mainly to produce mycotoxins. *Fusarium* spp. belongs to these fungi. *Fusarium graminearum (Gibberella zeae), F. avenaceum (G. avenacea),* and *F. culmorum* were the most widely spread species of grain crops in Europe in early 2000s (Bottalico & Perrone, 2002). These three species dominated in wheat grains from Denmark with a maximum accumulation of only one mycotoxin DON (Nielsen et al., 2011). *F. poae, F. tricinctum, F. sporotrichioides, F. equiseti,* and *F. langsethiae* were often also isolated (Bottalico & Perrone, 2002). The study of wheat grain samples from three regions of Russia (Volga, Ural, and West Siberia) has shown considerable dominance of *F. sporotrichioides* in 2017 (Gagkaeva et al., 2019). Since the early 2000s, following the audit of the *Alternaria* spp. (Simmons, 2007), most researchers have begun to identify its species from wheat seeds. Ten species of the genus were isolated on cereals. Most cereals are colonized by small-spore species: *A. arborescens, A. alternata, A. tenuissima*, and *A. infectoria*. The most widely spread information is about the distribution of the latter three species in Argentina (Andersen et al., 2015), Italy (Logrieco et al., 2009), Norway (Kosiak et al., 2004), Germany (Müller & Korn, 2013), and Russia (Gannibal, 2018).
Seed treatment is necessary for overcoming the harmful effects of seed and soil infection and for rapid germination. In Ukraine, wheat cultivation is not possible without the use of fungicides. Chemical agents with one active ingredient are actively replaced by two or three-component pesticides, thus overcoming pathogens' resistance to certain active substances. The main task of chemical agents is to control dangerous pathogens, which were shown in many scientific studies. However, their application has negative results: phytotoxic action, destruction of soil microflora, mycorrhizal fungi (Channabasava et al., 2015). The efficiency of seed treatment is determined even by a percentage of seed infection. Treatment of wheat and barley seeds with 12 fungicides showed the expedience of their use against infections *F. graminearum* (63%). The low infection rate of seeds (5–10%) did not affect grain germination or yields (May et al., 2010).
Seed microbiota is a variable system that needs monitoring. Solitary studies of different fungi at different times do not give a full understanding of complex microbial changes. One of the factors that regulate the microbiota is seed treatment. The effective action of pesticides is mostly directed against the most harmful and the most widely spread genus and species of phytopathogens. There is no information concerning other species. A known fact is the suppression of seed germination, which is explained by violations of pesticide and adverse weather conditions. Other reasons are not considered. Therefore studies of seed microbiota, its variability, and the effect of seed treatment on the microbial complex formation are quite relevant.

## Methods

The effectiveness of chemical and biological preparations was checked on the winter wheat seed of Bogdana variety. It was grown under conditions of North-East of Ukraine (Sumy oblast). The seed of three-year yields (2017-2019) was analyzed. The seed microbiota was studied using a biological method (Naumova, 1951).
Potato-glucose agar was used for the analysis. Sterilized Petri plates of 9 cm diameter were filled with agar medium. Twenty-five seeds were placed into each dish. As many as 200 seeds were used for each variant. Seeds were treated before they were placed into Petri dishes. Wheat seeds were soaked in sterilized water as a control treatment. After seven days of incubation in darkness under the temperature of 24 °C, fungi' amount and species composition were counted and identified. The identification of fungi was based on colony characteristics and morphology of mycelium and sporulation.
The seed was treated according to the producer's instruction: Maxim 0.25 FS from Syngenta with the consumption rate of 1.5 – 2l/t (active component Fludioxinil 25g/l), Rostock 50% from the company Agrarian Resource with the consumption rate of 1l/t (active components: Carboxyl 400g/l, Triadimenol 97g/l, Tebuconazole 3g/l), Kinto Duo from BASF with the consumption rate of 2 – 2.5l/t (active components: Prochloraz 60g/l, Triticonazole 20g/l), Phytosporin-M from OZHZ company with a consumption rate of 15g/l (Bacillus subtilis bacteria strain 26D, 100 mln cells/g). The amount of preparation per 0.5kg of winter seed was calculated.
The second step was to study the effects of seed germination preparations: the amount of germinated seeds (on the 7th day) and the length of seedlings (on the 7th and 14th days) were counted and measured. The fungicide's effect on seed germination into the soil was studied in the laboratory. Ten plastic cups with soil were taken for each variant; five seeds were planted into each of them.

## Results

**Influence of seed treatment on seed-born microbiota in North-Eastern Forest Steppe of Ukraine.** The effectiveness of the fungicidal treatment of wheat seed was performed during three years (2017–2019). The peculiarities of winter wheat microbiota formation were studied depending on pesticide action. It was found that fungicides significantly affected the isolation of fungi from seeds. Seed treatment does not always reduce the number of colonies and mostly affects fungi' qualitative composition, especially chemicals. Thus, only 6% of seeds did not have colonies when treated with Maxim 0.25 FS in 2019. Fungi sprouted from all seeds when treated with other fungicides. In 2017 a definition of fungi by cultural and morphological characteristics testified that small-spore fungi of genus *Alternaria* spp. prevailed in the mycobiota of winter wheat seeds in two variants: in control (57.8%) and the variant with the use of Fitosporin-M (72%) (Fig.1).

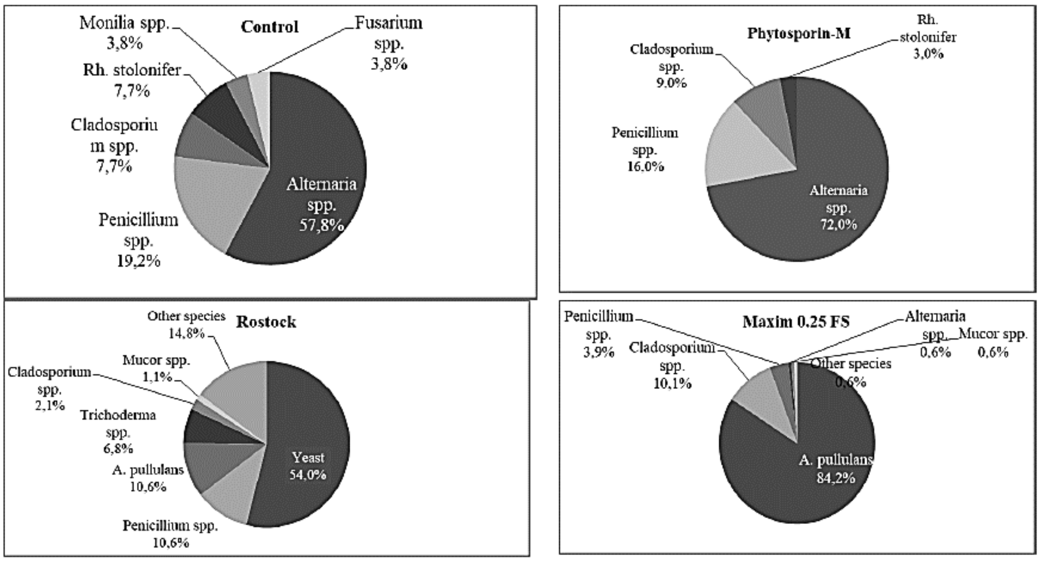


**Fig. 1.** Microbiota of winter wheat seed depending on the treatment in 2017 (% from the total amount of colonies) ($LSD_{05}$ *Penicillium* spp. = 1.2, $LSD_{05}$ *Cladosporium* spp. = 0.6).

In 2018 one more fungicide Kinto Duo was added to the experiment. A visual examination showed some colonies' similarity in variants with chemical preparations, while the similarity between control and variant with Phytosporin - M. *Alternaria* spp. was dominated in seed microbiota in 2018, like in 2017 (63.5%). A significant percentage of isolation had *A. pullulans* (20.6%) (Fig. 2).

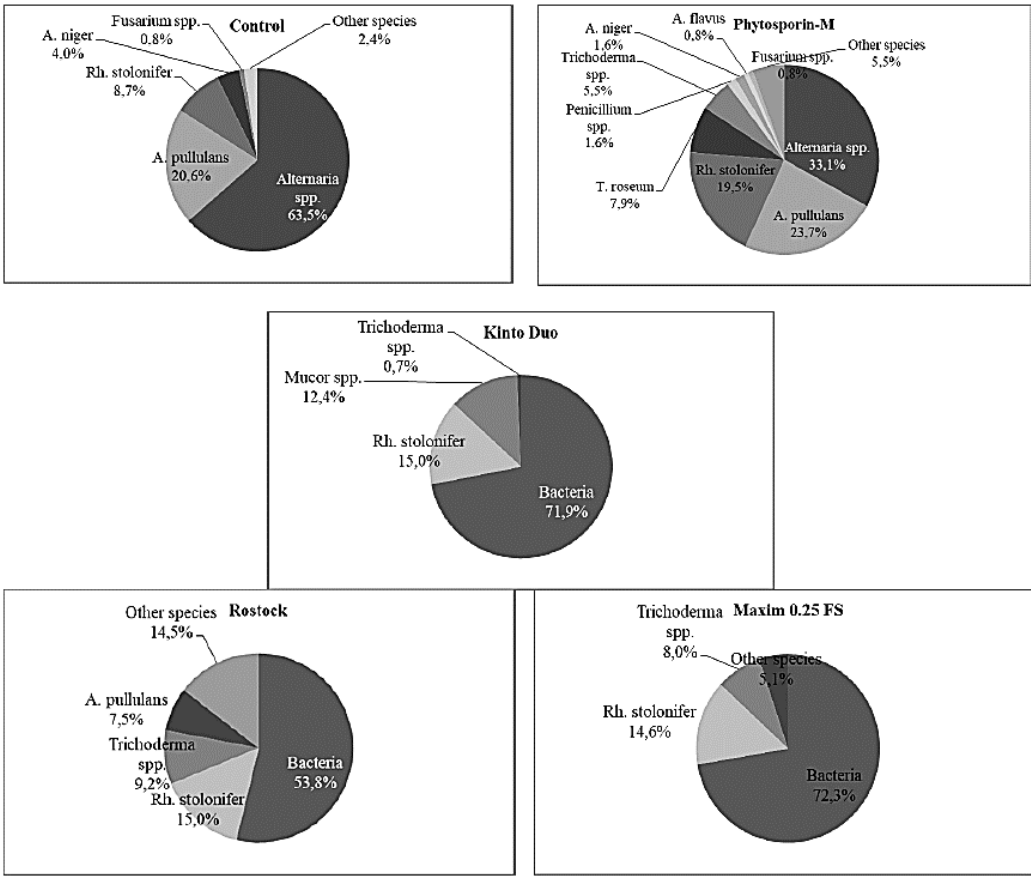


**Fig. 2.** Microbiota of winter wheat seed depending on the treatment in 2018 (% from the total amount of colonies). $LSD_{05}$ bacteria =3.9, $LSD_{05}$ other species = 2.8, $LSD_{05}$ *A. pullulans* = 2.1, $LSD_{05}$ *A. niger* = 1.5, $LSD_{05}$ other species=1.2, $LSD_{05}$ *Rh. stolonifer* = 2.2, $LSD_{05}$ *Alternaria* spp. = 4.9.

The percentage of *Penicillium* spp. was also significant (control –19.2%, Phytosporin-M – 16%). The use of chemical preparations led to the isolation of fungi that were not found in control. Yeast colonies (54%) dominated when treated with Rostock to treat Maxim 0.25 FS – *A. pullulans* (84.2%). Chemicals adversely affected the development of *Alternaria* spp. and reduced the amount of *Penicillium* spp. Instead of one fungus (*Rh. stolonifer*) from the subdivision of Mucormycotina was isolated another one (*Mucor* spp.) In a variant with Rostock was isolated *Trichoderma* spp.

Two species of *Aspergillus* spp. (*A. niger*, *A. flavus*) were isolated on the control and under treatment with Phytosporin-M. The use of Phytosporin-M expanded the range of fungi isolated from seeds. The amount of *Alternaria* spp. decreased twice. The amount of Fusarium spp. was like in control. Chemicals had the same influence on seed microbiota: bacterial colonies dominated in all three variants. An interesting fact was the isolation of *Trichoderma* spp. in seed-treated variants. The highest amount of these fungi was noted in the variant with the use of Rostock.

In 2019 a visual inspection revealed the presence of only fungi colonies in the variants. *A. pullulans* dominated in the mycoflora of winter wheat seeds (Fig. 3). On average, 19 colonies were extracted from each Petri dish in a variant with Maxim 0.25 FS seed treatment, with Rostock – 23, Phytosporin-M – 24, and 29 in control.

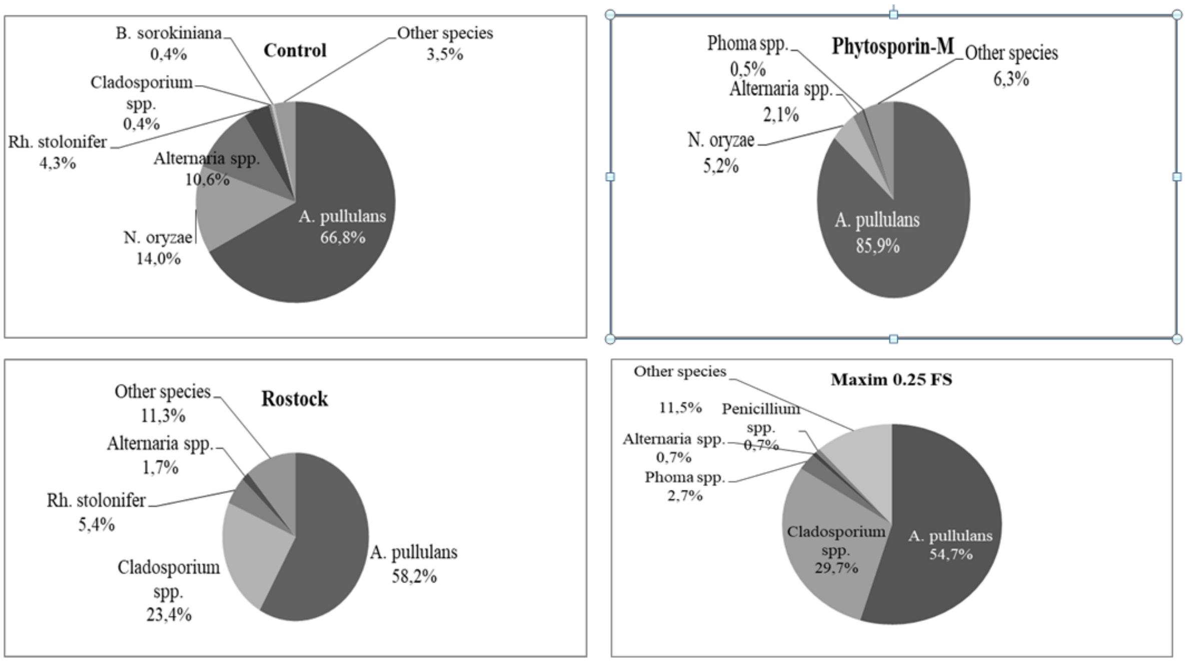


**Fig. 3.** Microbiota of winter wheat seed depending on the treatment in 2019 (% from the total amount of colonies) ($LSD_{05}$ *Alternaria* spp. = 0.5, $LSD_{05}$ other species=1, $LSD_{05}$ *A. pullulans* =0.5).

The 2019 wheat seed microbiota was the most different from the two previous years of research. *Alternaria* spp. was third as to isolation has given the first place to *Nigrospora* spp. Wheat seed did not germinate when infected with *B. sorokiniana*.

The most significant amount of *A. pullulans* was noted under seed treatment with Phytosporin-M – 85.9 % (Fig. 4). Biological seed treatment reduced the amount of *Nigrospora* spp. and *Alternaria* spp. several times. Chemical agents did not give germination of *Nigrospora* spp., reduced the amount of *A. pullulans*, *Alternaria* spp. Nevertheless, fungicidal seed treatment provoked the isolation of other dark-colored fungi – Cladosporium spp.

Thus, seed treatment has altered the winter wheat microbiota during three years of research; these microbiota changes varied by year. Biological seed treatment has caused less change than chemical treatment. Fungicides have replaced the dominance of *Alternaria* spp. on the prevalence of yeast and *A. pullulans* in 2017, bacteria in 2018, reduced *A. pullulans* in 2019.

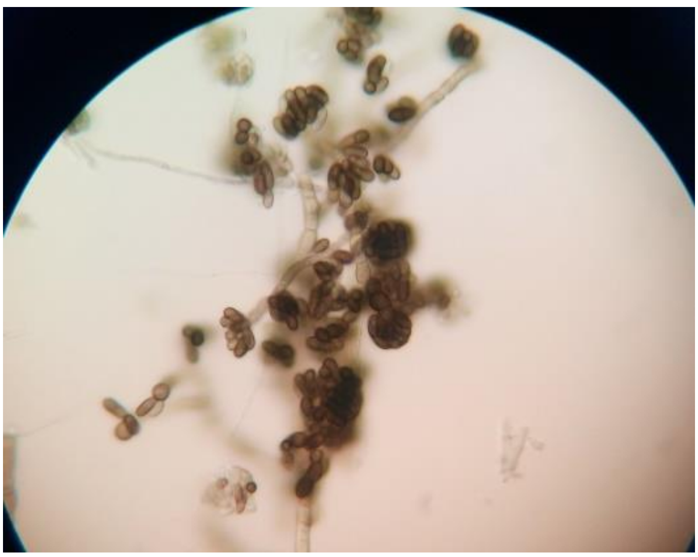

**Fig. 4**. Sporulation of A. pullulans in the variant with Phytosporin-M (2019).

**Effect of seed treatment on length of wheat seedlings.** The studies of the effectiveness of chemical agents showed further development of plants. It was found that seed treatment adversely affected the growth of winter wheat on the agar medium. The seedlings' length was measured on the 7th day in 2017–2019 and the 14th day in 2019 (Table 1).

**Table 1.** Effect of seed treatment on the length (mm) of winter wheat seedlings on the agar medium (2017–2019).

| Variant | 2017 | 2018 | 2019 | |
|---|---|---|---|---|
| | the 7th day | the 7th day | the 7th day | the 14th day |
| Control | 48 | 39 | 26 | 95 |
| Kinto Duo | - | 26 | - | - |
| Phytosporin-M | 57 | 40 | 27 | 120 |
| Maxim 0.25 FS | 18 | 25 | 13 | 51 |
| Rostock | 40 | 33 | 19 | 100 |
| $LSD_{05}$ | 5.8 | 5.9 | 1.7 | 7.9 |

The smallest seedlings were obtained in the variant with seed treatment Maxim 0.25 FS. Rostock also reduced the length of seedlings. Seedlings were longer with the use of Phytosporin-M than in control. In 2018, seeds were further treated with Kinto Duo, which also negatively impacted plant development. In 2019 the fungicide Maxim 0.25 FS had the most significant negative impact on seed germination. Twelve seeds from the tested seed (200) did not germinate on the 7th day, but they formed 2-3 roots. Two eeds did not form any germs under Rostock action. The seeds in all other variants germinated, except one with a colony of *B. sorokiniana*. Plant length was additionally measured on the 14th day in 2019. The results were identical. They were more contrast in the variant with Phytosporin-M than on the 7th day: the length of seedlings exceeded the control by 25 mm on the 14th day. The negative effect of Rostock decreased slightly (from 7 to 5 mm), and the negative effect of Maxim 0.25 FS remained almost similar – the lag of growth from control in 2 times.
A comparison of the length of seedlings by years showed its decrease from 2017 to 2019. The smallest plant length was noted in 2019 in all four variants. Such an ordinary picture of treated and untreated variants indicates that other factors except seed-born microbiota (perhaps wheat-growing conditions) influenced seed germination.
The negative impact of chemicals contributed to studying the effectiveness of seed treatment for sowing seeds into the soil. The plants were grown in the laboratory. The amount of germinated plants, the length of seedlings, and the aerial phytomass were determined on the 14th day (Table 2).

**Table 2.** The impact of fungicides on the germination and development of wheat plants in the soil (2017).

| Variant | The amount of germinated plants | Length of seedlings, cm | Phytomass of the aboveground part of plants, g |
|---|---|---|---|
| Control | 46 | 9.91 | 7.79 |
| Phytosporin-M | 49 | 9.57 | 8.36 |
| Maxim 0.25 FS | 47 | 8.09 | 6.85 |
| Rostock | 47 | 8.55 | 7.48 |
| $LSD_{05}$ | - | 2.20 | - |

A phytotoxic effect of preparations was lower, but a common tendency remained: biological preparation stimulated and chemical agents delayed the development of wheat plants. Biological preparation Phytosporin-M promoted the most considerable amount of seeds in the soil and allowed to form the most significant aboveground mass. While chemicals helped more plants germinate than in control, but they adversely affected plant phytomass.

## Discussion

In Ukraine, microbiota studies of winter wheat seeds were mostly random, conducted in some regions and with different intervals. The latest research was conducted on the distribution of *Fusarium* spp. and *Alternaria* spp. all over the country. Analysis of grain material from different Ukraine regions showed a high percentage of infection with fungi, although the wheat crop was 2-3 times treated with fungicides. 44.6 % of *Alternaria* spp. and 38.4 % of *Fusarium* spp. were isolated from wheat seeds from Sumy oblast in 2015 (Mykhalska et al., 2019). Seven *Fusarium* species were identified in winter wheat seed: *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. langsethiae*, *F. poae*, *F. sporotrichioides*, *F. tricinctum*, while *F. graminearum* dominated in all regions of Ukraine. Only two *Fusarium* species (*F. graminearum* – 66.6 % and *F. avenaceum* – 33.4%) were identified in wheat seed samples from Sumy oblast (Hrytsev et al., 2018).
Our research of seed microbiota showed a high percentage of *Alternaria* spp. (10.6 – 63.5 %) infection and a low presence of Fusarium spp. (0.8–3.8 %) in control (Sumy oblast). The fungus complex of winter wheat seeds was very variable during three years in the control variant. In 2017 seed microbiota consisted of 7 species, one of which dominated (Alternaria spp). Penicillium spp. ranked second in infection. Other fungi were not so spread (*Cladosporium* spp., *Rh. stolonifer*, *Monilia* spp. and *Fusarium* spp.). In 2018 more than five species were found in the seed complex of fungi. *Alternaria* spp. also dominated, but A. pullulans were second in infection. Alongside other species, a new one was revealed– A. niger. In 2019 more than six species were isolated in seed microbiota. The amount of *Alternaria* spp. decreased to 10.6%. A. pullulans occupied a dominant position among other fungi. A new species (*N. oryzae*) appeared in a large amount. The presence of the most harmful species (*B. sorokiniana*) was

noted. The amount of genera and species of wheat seed mycoflora depends on the number of samples, their location, the area and technology of crop cultivation, varieties, methods of research, and human factors. In Pakistan, 80 wheat samples from four different geographical areas were analyzed. Scientists have identified three genera (*Fusarium* spp. (42%), *Drechslera* spp. (35%), *Phytophthora* spp. (16%)) and two species (*Alternaria alternata* (49%), *Aspergillus niger* (46%)) in the microflora (Ur-Rahman et al., 2018). One hundred twenty wheat samples (12 varieties) were examined, and five fungus species were identified (*Alternaria tenuis*, *Aspergillus niger*, *Fusarium moniliforme*, *Curvuluria lunata*, and *Stemphylium herhurum*) from one province of Pakistan (Rajput et al., 2005).

The results of seed treatment effectiveness are mainly related to some genera and species of fungi microbiota. They depend on the choice of methodology. For example, analysis of seed treatment effectiveness on a nutrient medium showed a smaller reduction in the amount of *Alternaria* spp., *Fusarium* spp., and *Helminthosporium* spp. than on rolls of paper. All six fungicidal chemicals reduced these fungi from 22 to 60% on the nutrient medium. Two chemicals were the most effective: Kinto Duo (2.5 l/t) and Polaris (1.5 l/t). Dividend Extreme (0.75 l/t) reduced the amount of *Alternaria* spp. from 89 to 55%, and *Fusarium* spp. – from 9 to 8 % (Zheltova & Dolzhenko, 2016). In the same year (2011), a Dividend Extreme efficiency study with a slightly higher rate (0.8 l/t) on filter paper rolls was conducted. The biological effectiveness of this fungicide was 100% (Polunina et al., 2017).

The phytotoxic effects of fungicides were shown in many scientific studies. Therefore our findings do not contradict most of the results. Phytotoxic effects were proved when studying the chemical substances' influence on germination energy and laboratory germination of winter wheat seeds even in the recommended doses. Five of the six fungicides reduced germination energy: Dividend Extreme decreased this indicator by 39.5%, Maxim – by 30.5%, Serticor – by 13.5%, Celest Top – by 10.5%, Dividend Star –by 7.5% as compared to control. 4 pesticides reduced laboratory germination of winter wheat seeds: Certicor by 14%, Maxim and Dividend Star by 3%, Dividend Extreme by 2% (Pavlyuk & Shentsev, 2016).

Investigation of three active substances (prochloraz, prothioconazole, cyproconazole) of modern fungicides for seed treatment in different doses showed a significant phytotoxic effect on wheat. For example, prochloraz (Kinto Duo) had the least impact on the development of wheat plants. For 300 μg/10 seeds, the length of seedlings and roots was the smallest 45.4 mm and 25.8 mm, for 200 μg/10 seeds – 68.8 mm and 46.9 mm, for 100 μg/10 seeds – 58.7 mm and 39.9 mm. While in control, these characteristics were 93.5 mm and 43.9 mm, respectively (Baybakova et al., 2016).

The stimulating effect of Phytosporine-M is a proven fact, and our studies have confirmed it like some others. For example, Phytosporin-M was used to find new effective strains for biological preparations that would be effective at low temperatures. The germination energy of spring wheat seeds in an intact control was 29%, in a variant with Phytosporin-M – 76%. Laboratory germination in the first case was 83%, in the second – 97%. Plants developed better under the biological preparation influence than in the intact control: the length of seedlings was 8.05 cm longer and roots 4.26 cm longer (Subbotin et al., 2016).

## Conclusions

The variability of the microbiota of winter wheat seed during the three-year studies under conditions of North-Eastern Ukraine (Sumy oblast) has been proved. In 2017–2018 small-spore *Alternaria* spp. dominated, and in 2019 – A. pullulans. Fungicides have significantly changed the winter wheat microbiota during three years of research. In some cases, they decreased the number of microorganism colonies and sometimes even increased. Qualitative changes in the composition of seed microbiota were noted. Chemical preparations inhibited the development of *Alternaria* spp. Phytosporin-M reduced the amount of fungus *N. oryzae* (which adversely affects the germination of seeds) and *Alternaria* spp., except in 2017. Fungicides (Maxim 0.25 FS, Rostock 50%, Kinto Duo) inhibited seed germination and seedling development on agar medium and soil, whereas Phytosporin-M promoted wheat germination and greatly exceeded control.

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