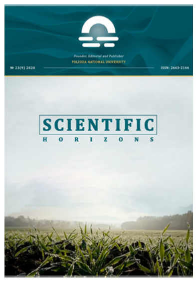
SCIENTIFIC HORIZONS
Journal homepage: https://sciencehorizon.com.ua/en
Scientific Horizons, 25(4), 45-52



UDC 582.282: 633.11
DOI: 10.48077/scihor.25(4).2022.45-52

Linear Growth of Representatives of Wheat Seeds Mycobiota

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Article's History:
Received: 30.04.2022
Revised: 29.05.2022
Accepted: 30.06.2022

Suggested Citation:
Rozhkova, T., Golosna, L., Afanasieva, O., Nemerytska, L., & Zhuravska, I. (2022). Linear growth of representatives of wheat seeds mycobiota. *Scientific Horizons*, 25(4), 45-52.

Abstract. Seed-born fungi of wheat interact with the plant at various stages of its development and with each other. With the highest growth rate, they should be isolated because of competition. The purpose of this study was to compare the growth of colonies on a nutrient medium for the gradation of fungal genera and species from wheat seeds according to aggressiveness. These data helped concluding on the effectiveness of mycoexpertise of winter wheat seeds. Potato-glucose agar (PGA) was used for the analysis of the fungal complex. Seven-day fungal cultures were sown in the centre of Petri dishes. Linear growth of fungal colonies on PGA with gentamicin was determined. The specific features of the development of 12 representatives of seeds mycobiota from the Northeast of Ukraine of the 2017-2019 harvest were investigated. The dominance of *Alternaria* sp. and a slight release of *Fusarium* sp. were established by analysis of the fungal complex. The first comparison of the linear growth of *Fusarium graminearum*, *F. poae,* and *Alternaria tenuissima* in 2017 showed that *Fusarium* colonies grow faster on nutrient medium. In 2018, the growth characteristics of *A. arborescens*, which quickly became dominant in wheat seeds mycoflora, and the little-common *Trichothecium roseum* were studied in detail. By comparing the growth of fast-growing *F. graminearum* with the common *Aureobasidium pullulans* and the aggressive *Nigrospora oryzae*, the fastest development of the third and the slowest of the second species was established. *F. poae* filled the Petri dish on day 6, *Penicillium* – on day 22. In 2019, in the first experiment comparing *F. poae, F. sporotrichioides,* and *A. avenicola*, the second species had the worst growth rates. It became the second fastest growing colony in the study of the growth of seven species in the second experiment. Isolates of *N. oryzae* in 2018 were more aggressive than in 2019. Specific features of colony growth on PGA did not affect the effectiveness of the analysis of mycobiota of winter wheat seeds. *N. oryzae* had the highest radial speed under the general dominance of *Alternaria* sp. *Fusarium* sp. (*F. poae, F. sporotrichioides, F. verticillioides,* and *F. graminearum*) and *B. sorokiniana* developed rapidly. *A. arborescens* and *A. avenicola* grew at the same level as *A. pullulans*. *Penicillium* and *T. roseum* lagged behind other fungi in speed and filled Petri dishes for the longest time. *A. tenuissima* had the lowest radial growth rate

Keywords: fungal seed complex, colony growth, potato-glucose agar, winter wheat

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INTRODUCTION

The mycobiota of wheat seeds is a dynamic system comprising varied species of fungi. Its composition is determined by abiotic and biotic factors. The seeds contain fungi that do not have phytotoxic effects on the plant (endophytes) and phytopathogenic species. The negative effects of phytopathogens are associated with the production of secondary metabolites that are dangerous to plants, animals, and humans. The pathogenicity of fungi is because they produce phytotoxins, enzymes, effector proteins, etc. (Peng *et al.*, 2021). The composition of seed mycobiota includes fungi that contaminate agricultural products with mycotoxins. These are *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp., *Alternaria* sp., *Cladosporium* sp., *Cochliobolus* sp. etc. Mycotoxins contaminate 20-25% of food crops in the world (Eskola *et al.*, 2020). Studies on the co-cultivation of fungi and bacteria revealed the synthesis of some of these metabolites, which allowed researchers to determine the cause of mycotoxins – competition between different representatives of the plant microbiome (Venkatesh & Keller, 2019). The endophytic microbiota is associated with the host plant throughout its ontogenesis. Endophytes have a positive effect on plants, increasing drought resistance and resistance to pathogens, stimulating plant growth and development (Hardoim *et al.*, 2015; Shahzad *et al.*, 2018; Kuźniar *et al.*, 2020).

The study of mycobiota in Ukraine, as in the world, is not permanent, is not included in state monitoring, but only depends on the scientific interest of researchers. For the last 20 years, Ukrainian scientists isolated and identified the following genera in the from wheat seeds*: Acremoniella* sp., *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Cochliobolus* sp., *Curvularia* sp., *Epicoccum* sp., *Fusarium* sp., *Mucor* sp., *Nigrospora* sp., *Penicillium* sp., *Phoma* sp., *Stemphylium* sp., and *Sordaria* sp. A study of 70 samples of wheat grain harvested in 2016 and 2017, collected in collective farms, the private sector, elevators, breeding stations, and regional seed inspections of three zones of Ukraine showed that 1 g of wheat grain in Ukraine contained from 1.12x103 to 6.5x104 CFU, which averaged to 3.3x104 ± 3.2x104. Inside the seeds were 11 representatives of the fungal complex. The most common were *Alternaria* spp. (67% of samples), *Aspergillus* spp. (37%), *Phoma exiqua* (30%), less frequently identified *Fusarium* spp. and *Mucor* spp. (in 19% of samples) (Ostrovskyi *et al.*, 2018). The 2016-2017 analysis of fungi isolated from wheat seeds of two varieties (Levada and Podolyanka) in Poltava Oblast showed the presence of 8 species/genera: *Alternaria alternata* (Fr.) Keissl., *Tilletia caries* (DC.) Tul. & C. Tul., *Fusarium* spp., *Cladosporium herbarum* (Pers.) Link, *Bipolaris sorokiniana* (Sacc.) Shoemaker, *Mucor* spp., *Penicillium* spp., and *Aspergillus* spp. Among the fungi of the mycoflora, only one species dominated – *A. alternata* (Pospelov *et al.*, 2020). The study of mycobiota of wheat seeds from the Right Bank Forest-Steppe of Ukraine from different varieties showed the presence of the following fungi: *Alternaria tenuis* Nees, *Fusarium graminearum* Schwabe, *Nigrospora oryzae* (Berkeley et Broome) Petch., *Aspergillus niger* Tieghem, Penicillium Link. The highest intensity of spore formation was observed in *A. niger* and *Penicillium* – from 3.2 to 12 million units/ml. High intensity of spore formation was inherent in *A. tenuis* and *F. graminearum* species and ranged from 1.4 to 7.2 million units/ml. (Mostovyak *et al.*, 2020). Analysis of wheat seeds with black point grown in Kyiv Oblast in 2018-2019 allowed identifying 13 species from 9 genera: *Alternaria, Fusarium, Curvularia, Bipolaris, Aspergillus, Acremoniella, Stemphylium, Sordaria,* and *Epicoccum*. *Alternaria* fungi dominated: *A. tenuissima* (Nees & T. Nees: Fr.) Wiltshire, Trans., and *A. infectoria* E.G. Simmons were most often isolated (Golosna, 2021).

The species composition of fungi mycobiota seeds determines the spectrum of secondary metabolites. Ukrainian scientists identify species of the most harmful and common representatives of the seed complex of fungi. The first representatives include belonging to *Fusarium* sp., the second – to *Alternaria* sp. According to the latest research, seven species of fungi of the genus *Fusarium* have been identified in 109 samples of winter wheat seed material from 78 districts of 21 regions of Ukraine: *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith) Sacc., *F. graminearum*, F. *langsethiae* Torp & Nirenberg, *F. poae* (Peck.) Wollenw., in Lewis, *F. sporotrichioides* Sherb. and *F. tricinctum* (Corda) Sacc. *F. graminearum* was the most common species in the country (the share of detection was 71%) (Gritsev *et al.*, 2018). Analysis of isolates of the genus *Alternaria* from different regions of Ukraine during 2012-2013 showed the dominance of *A. tenuissima* (70%) and a considerable percentage of *A. infectoria* (25.6%) (Golosna, 2015).

Mycobiota fungi of wheat seeds interact not only with the plant, but also with each other. Admittedly, all the features of their interaction *in vivo* may be unknown, but *in vitro* studies provide insight into some of them. Therefore, *the purpose of this study* was to compare the growth of colonies on agar medium to understand the effect of fungal aggressiveness on the composition of mycobiota of winter wheat seeds.

MATERIALS AND METHODS

The study was conducted during 2017-2019. Mycobiota fungi of winter wheat seeds were isolated from 43 samples obtained from agricultural enterprises of different districts and scientific institutions of the North-East of Ukraine. The authors of this study grew some wheat in the conditions of educational and scientific production complex of Sumy National Agrarian University. Before the analysis, the seeds (200-400 from the sample) were washed under running water for one hour, disinfected with 1% potassium permanganate solution for 1-2 minutes. The seeds were spread on a potato-glucose agar. 25 seeds were placed in one Petri dish. Petri dishes were incubated for seven days in a thermostat at a temperature of 20°C for germination of fungal colonies. Species were identified by various scientific studies: *Fusarium* sp. – by Leslie & Summerell (2006), Gagkaeva *et al.* (2011); *Alternaria* sp. – by Hannibal (2011), Woudenberg *et al.* (2013); *Aureobasidium pullulans* (de Bary)

G. Arnaud – by Zalar *et al.* (2008); *Nigrospora oryzae* (Berkeley et Broome) Petch. – by Wang *et al.* (2017); *Trichothecium roseum* (Pers.) Link – by Watanabe (2002); *B. sorokiniana* – by Manamgoda *et al.* (2014). The identified fungi were seeded in pure culture on PGA with gentamicin. Mycelial growth was determined by growing fungi in Petri dishes on PGA. For this, a seven-day growing was involved. The fungi were placed with a needle at the centre of dishes. Colonies grew in a thermostat at 20°C, 22°C, 23-24°C. The incubation period depended on the growth characteristics of the fungi (7-25 days). To identify the linear growth, the diameter of the colonies was measured in two perpendicular directions. The radial growth rate of colonies was determined according to the formula (1) (Poliksenova *et al.*, 2004):

$$Kr = (r - r_0)/t \quad (1)$$

where Kr is the radial growth rate of colonies, mm/day; r is the radius of the colonies at a given time, mm; t is the time from sowing to the moment when the colony will have a radius r, day.

Repetition depended on the experiment: three to five times. Statistical analysis of the results was performed according to the method of one-way analysis of variance in Excel, calculating the LSD_{05} according to Dospekhov (1985).

RESULTS AND DISCUSSION

According to the authors' observations, *Alternaria* fungi dominated the mycoflora of winter wheat seeds grown in the North-East of Ukraine (2021). *Fusarium* fungi were quite rare. Usually, one infected grain of this genus was found in a Petri dish. In 2017, the authors of this study concluded that *in vitro* conditions are better for the development of *Alternaria* sp. than Fusarium sp. The definition of *Alternaria* sp. recommends using Potato-Carrot Agar (PCA), Hay Infusion Agar (HAY), and V-8 (Vegetable Juice Agar) (Gannibal, 2011). *Fusarium* sp. is better determined on Carnation Leaf-piece Agar (CLA), Spezieller Nährstoffarmer Agar (SNA), and Potato Dextrose Agar (PDA) (Leslie & Summerell, 2006).

Therefore, the growth of fungi was investigated on the PGA medium (Table 1). The number of repetitions was 5 times.

***Table 1**. Growth of mycelium on PGA medium (20°C, 2017)*

Fungal species	The diameter of the colony, mm		
	Day 3	Day 4	Day 7
F. graminearum	17.4x15.8	24.8x25	51.7x55
F. poae	10x10	22.3x21.3	52.5x49.3
A. tenuissima	7x7.8	12.4x11.4	33.8x33.8
LSD_{05}	1.8	4.1	3.4

The assumption turned out to be wrong, which is confirmed by the data from Table 1. *Fusarium* fungi grew much faster on the medium than *A. tenuissima*. If *Fusarium* sp. indeed were present in a batch of grain in considerable quantities, they would quickly inhibit the development of *Alternaria* fungi, which was observed in 2016 on the Samuray variety. *F. graminearum* had higher colony growth rates on PGA than *F. poae*. When studying the growth rate of six species of *Fusarium* fungi on the KGA, the highest rate was found in *F. graminearum* – 23.6 mm/day. *F. poae* has also been classified as a fast-growing species. Its growth rate was 21.7 mm/day (Shashko, 2020).

In 2018, the authors of this study decided to investigate the specific features of the growth of fungi on a nutrient medium in more detail. *A. arborescens* E.G. Simmons appeared unexpectedly and quickly dominated the mycoflora of wheat seeds. The specific features of the growth of this species on a nutrient medium were investigated (Fig. 1).

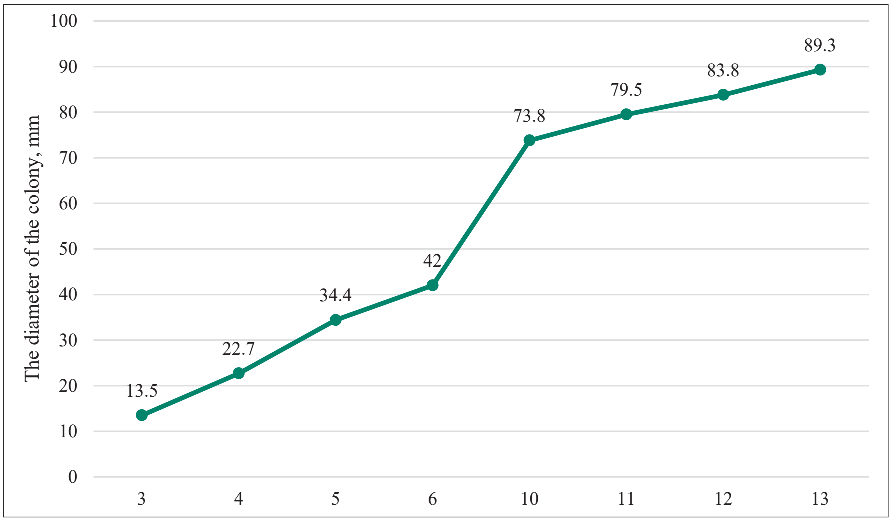

***Figure 1**. The diameter of A. arborescens (growing at 22°C) (2018)*

This species developed quite rapidly compared to other *Alternaria* fungi. On days 13-14, the fungi colony completely filled the Petri dish. The average radial growth rate was 3.4 mm/day. By Day 3, the fungi developed slowly. From Day 4, the active development of the fungal colony began. Its maximum growth rate was observed on Day 5. Then the figure started to gradually decrease. The lowest growth rate of the colony was observed on Day 12 of fungal development.

The occurrence of *A. pullulans* in 2016 was insignificant. Gradually, the amount of these fungi increased. Therefore, it was interesting to investigate their behaviour on medium without other fungi. During the isolation of fungi from the seeds, *N. oryzae* had increased aggression compared to other fungi. If they germinated from seed, no fungal colonies developed with them. Therefore, these fungi would develop separately on the medium. Thus, the authors started investigating the growth of fungi on the PGA (Table 2). Repeatability – three times.

***Table 2**. Comparison of growth of F. graminearum, A. pullulans, and N. oryzae (2018)*

Fungal species	The diameter of the colony, mm				
	Day 3	Day 6	Day 7	Day 8	Day 13
F. graminearum	21x21	55x52	65x62	77x73	90x90
A. pullulans	23x13	57x30	53x36	57x39	59x48
N. oryzae	46x43	90x90	90x90	90x90	90x90
LSD_{05}	3.5	3.9	6.3	4.9	4.7

N. oryzae filled the entire Petri dish on Day 6. This species proved to be the most aggressive, having the fastest growth of its colony. *A. pullulans* lagged behind the other two fungi.

The study of the growth of *Penicillium* fungi was complicated by the fact that over time, several colonies formed on the medium. Thus, out of five replicates of one colony on Day 4 of observation, 3 colonies were formed in one of the replicates, on Day 6 there were already two replicates with several colonies, on Day 11-3 replicates. Therefore, the data was analysed from only two replicates. The specific features of growth of a fairly common *Fusarium* species – *F. poae* (Fig. 2) were studied.

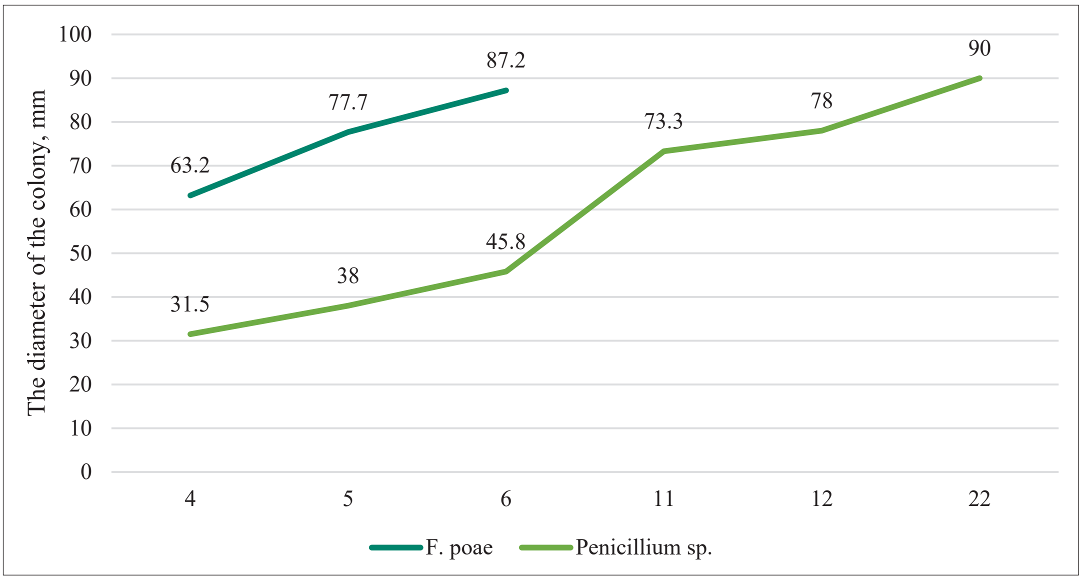

***Figure 2.** Linear growth of F. poae and Penicillium on PGA (2018) (LSD_{05}6=9.3)*

F. poae fungi demonstrated rapid colony growth: on Day 6, they filled almost the entire diameter of the Petri dish. Isolates of these fungi from wheat seeds in Poland on Days 4 and 7 of cultivation had a growth rate of 5.4-10.3 mm/day (Lukanowski *et al.*, 2008). On Day 4, the isolate under study had a speed of 15.8, and on Day 6-14.5 mm/day in the diameter of the colony.

The *Penicillium* fungi were inferior to the growth of the *Fusarium* fungi, but in the initial stages of development grew faster than *Alternaria* fungi. However, after Day 12 of cultivation, the rate of development of the fungi decreased. Only on Day 22 the fungi completely filled the diameter of the Petri dish.

Comparison of the growth rate of endophytic and phytopathogenic isolates of *F. poae*, *Alternaria alternata* and *Penicillium funiculosum* Thom on PGA arranged them in the above order. That is, the *Fusarium* fungi formed the fastest growing colonies. Phytopathogenic isolates had higher growth rates than endophytic ones (Kurchenko *et al.*, 2015).

T. roseum began to be observed in grain batches at the beginning of this study. Moreover, its presence was different: from isolated cases to a recurrence in the mycoflora of wheat seeds in 2020. These fungi behaved quite aggressively towards other fungi when they actively germinated from seed. Sometimes they co-existed with other fungi (mostly *Alternaria*), and it was even difficult to spot. Therefore, it was necessary to investigate their growth on the medium (fourfold repetition) (Fig. 3).

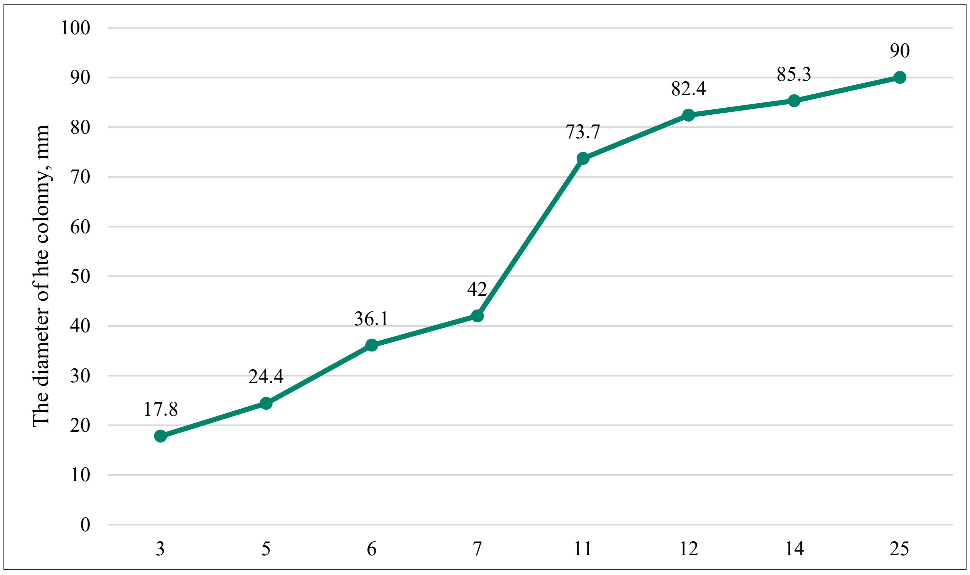

Figure 3*. The diameter of T. roseum (growing at 22°C) (2018)*

First, the gradual linear growth of the fungal colony was noted, which lasted for 12 days of their cultivation. They grew the fastest on Days 5-6 – 11.7 mm. After Day 12, the growth of the fungal colony started to slow down. Observations of the colony growth showed that it hardly grew. Only on Day 25 *T. roseum* completely filled the entire Petri dish. Isolates grown from winter wheat seeds harvested in 2019 were investigated in two experiments of single cultivation. First, the growth characteristics of the three following species were compared: *F. poae, F. sporotrichioides,* and *A. avenicola* (repeatability – four times) (Fig. 4).

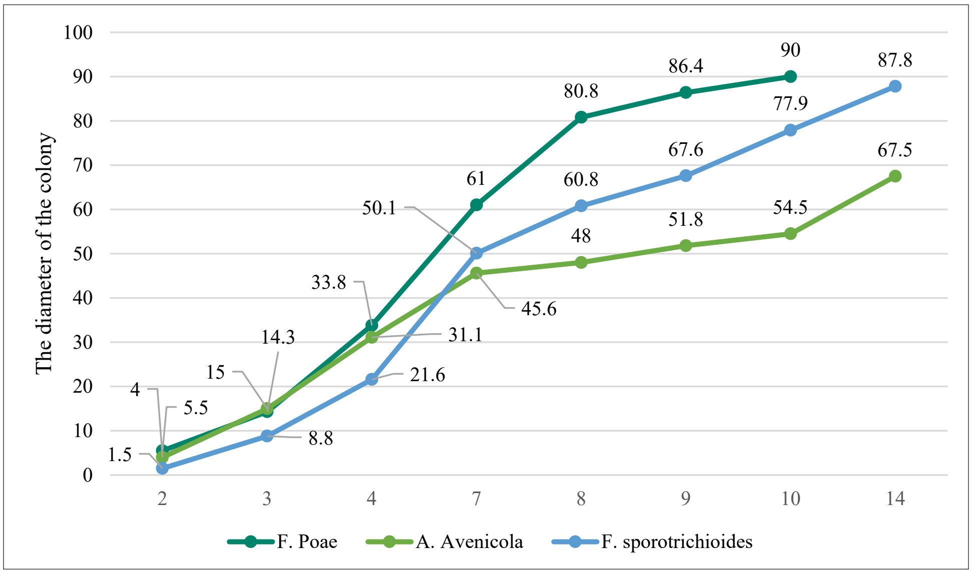

Figure 4*. Linear growth of F. poae, F. sporotrichioides, and A. avenicola (2019) (*LSD_{05}*7=4.5,* LSD_{05}*14=3.2)*

Isolates of *F. poae* grown from seeds of the 2019 harvest were less aggressive than their respective isolates of 2018 (Fig. 2). It took them 10 days to fill the entire surface of the medium. *Alternaria* species initially lagged behind *Fusarium* fungi, but from Day 7 it overtook the growth of the colony of *F. sporotrichioides*. Of the three species under study, the most aggressive was *F. poae*.

Simultaneous study of seven varied species of seeds mycobiota allowed isolating new fungi with rapid colony growth and confirm the high rate of already identified aggressive species (Table 3) (repeatability – three times).

***Table 3**. Comparison of the growth of fungal colonies of winter wheat seeds mycobiota (2019)*

Fungal species	The diameter of the colony for a certain day of cultivation, mm						
	Day 4	Day 6	Day 7	Day 11	Day 14	Day 19	Day 22
N. oryzae	43.7	84.7	90	-//-	-//-	-//-	-//-
F. sporotrichioides	43.2	69.2	77.7	90	-//-	-//-	-//-
F. poae	36.2	54.8	62.8	90	-//-	-//-	-//-
F. verticillioides	35.3	52.7	60	84.7	90	-//-	-//-
A. avenicola	24.5	43.5	47.2	80.7	90	-//-	-//-
A. arborescens	30.7	45.3	53.2	76.2	83.3	90	-//-
B. sorokiniana	37.7	53	61.5	75.3	77.2	83.5	87.5
HIP_{05}	2.5	3.4	3.1	2.6	Did not count		

In 2019, the fastest development of *N. oryzae* was confirmed. In 2018, isolates of these fungi were more aggressive; they completely filled the Petri dish on Day 6. Different ability of colonies of isolates of one species – *F. sporotrichioides* – was noted. If in the first experiment this species lagged behind the *Alternaria* fungi, then in the second – they were the second most aggressive species, even surpassing the fairly fast species of *F. poae*. The last fungi had a lower growth rate both compared to the first experiment and the previous year of the study. *F. verticillioides* predominated in the development of *Alternaria* fungi. *A. avenicola* grew faster from *Alternaria* species. *B. sorokiniana* developed seven days faster than *Alternaria* fungi and *F. verticillioides*, and from Day 11 their growth rate was minimal. Isolates of *B. sorokiniana* from barley seeds in Argentina had an average growth rate per PGA of 9.9 mm/day (Dominguez *et al*., 2020). The isolates under study had a colony growth rate of 8.8 mm/day on the seventh day.

Since mycobiota were analysed on Day 7, the radial growth rate during this period was calculated (Fig. 5).

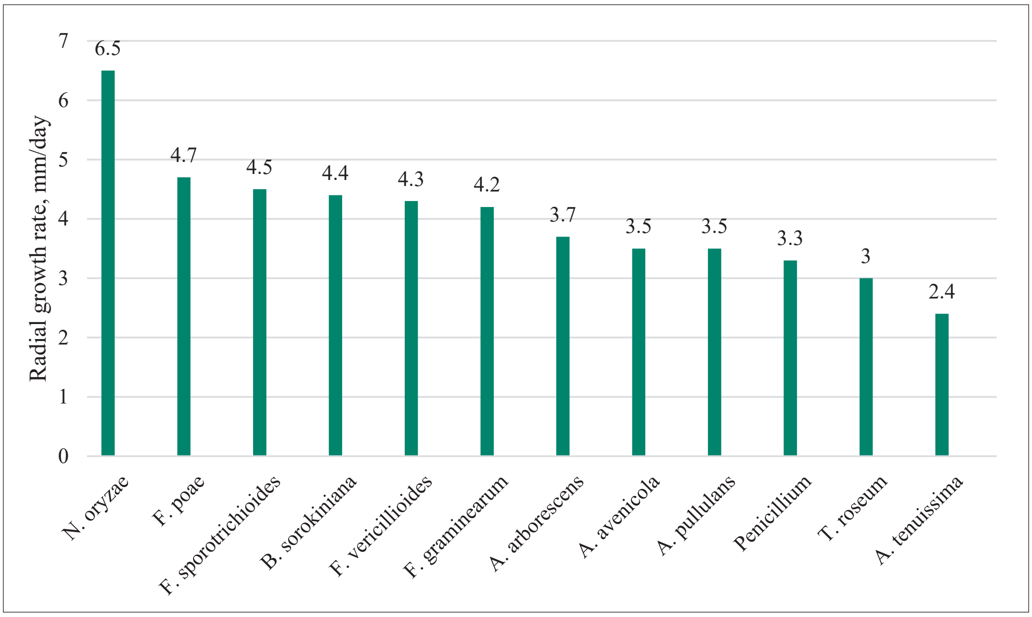

***Figure 5**. Radial growth rate of representatives of wheat seeds mycobiota (2017-2019)*

The area of variation of the indicator was 2.4-6.5 mm/day. *N. oryzae* had the maximum speed. Radial growth of *Fusarium* sp. was similar to *B. sorokiniana*. The next block in speed was *Alternaria* sp. and *A. pullulans*. *Penicillium* and *T. roseum* gave way to them. *A. tenuissima* had the lowest radial speed.

CONCLUSIONS

Single cultivation fungi of wheat seeds mycobiota on PGA medium showed different growth rates of colonies of isolates of different years, from different samples, but allowed distributing fungi by growth rate, i.e., aggressiveness. *N. oryzae* had the fastest development of colonies, followed by Fusarium *sp. (F. poae, F. sporotrichioides, F. verticillioides, F. graminearum),* and *B. sorokiniana* (only in the first seven days), which were inferior to *A. arborescens* and *A. avenicola*. *A. pullulans* developed at the level of *Alternaria* sp. The *Penicillium* fungi had average growth rates in the first week, but their growth rate gradually decreased. *T. roseum* developed similarly but had maximum time to fill the Petri dish. *A. tenuissima* showed the lowest radial growth rate. The growth of colonies on the PGA did not affect the specific features of the isolation of fungi from the seeds of winter wheat. Data on the isolation of fungi from mycobiota correlate with their presence inside the seeds and were not determined by their development on agar medium.

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Лінійний ріст представників мікобіоти насіння пшениці

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Анотація. Гриби комплексу насіння пшениці взаємодіють з рослиною на різних етапах її розвитку та між собою. Можливо, у результаті конкуренції краще виділяються гриби з найвищою швидкістю росту. Метою досліджень було порівняти ріст колоній на поживному середовищі для градації грибних родів та видів з насіння пшениці за агресивністю. Ці дані допоможуть зробити висновки про результативність мікоекспертизи насіння пшениці озимої. Аналіз грибного комплексу провели на картопляно-глюкозному агарі. Семиденні культури грибів висіяли у центр чашок Петрі. Визначили лінійний ріст колоній грибів на КГА з додаванням гентаміцину. Було вивчено особливості розвитку 12 представників мікобіоти насіння з Північного Сходу України врожаїв 2017–2019 рр. За аналізу грибного комплексу встановили домінування *Alternaria* sp. та незначне виділення *Fusarium* sp. Перше порівняння лінійного росту *Fusarium graminearum*, *F. poae* та *Alternaria tenuissima* у 2017 р. показало, що фузарієві колонії швидше ростуть на середовищі. У 2018 р. детально вивчили особливості росту *A. arborescens*, який швидко зайняв домінуюче положення у мікофлорі насіння пшениці, та малопоширеного *Trichothecium roseum*. За порівняння росту швидкоростучого *F. graminearum* з поширеним *Aureobasidium pullulans* та агресивним *Nigrospora oryzae* встановили найшвидший розвиток третього та найповільніший другого виду. *F. poae* заповнив чашку Петрі на шосту добу, *Penicillium* — на 22-гу. У 2019 р. у першому досліді за порівняння *F. poae, F. sporotrichioides* та *A. avenicola* другий вид мав найгірші показники росту. У другому досліді він став другим за швидкістю розвитку колонії при дослідженні росту семи видів. Ізоляти *N. oryzae* у 2018 р. були агресивнішими, ніж у 2019 р. Ріст колоній на КГА не вплинув на виділення грибів з насіння пшениці озимої. За загального домінування *Alternaria* sp. найвищу радіальну швидкість мав *N. oryzae*. Швидко розвивались *Fusarium* sp. з (*F. poae, F. sporotrichioides, F. verticillioides* і *F. graminearum*) та *B. sorokiniana*, *A. arborescens* та *A. avenicola* росли на рівні з *A. pullulans, Penicillium* та *T. roseum* за швидкістю відставали від інших грибів і найдовше заповнювали чашки Петрі. *A. tenuissima* мав найменшу радіальну швидкість росту

Ключові слова: грибний комплекс насіння, ріст колоній, картопляно-глюкозний агар, пшениця озима