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ORIGINAL ARTICLE

Interaction of endophytic fungi of winter wheat seeds

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Abstract

Seed endophytes are potential bioagents for plant protection and growth promoters. The question of the specifics of their isolation in cultural environments is not clear. The purpose of this study was to establish the nature of the interaction of endophytic fungi of wheat seeds with different levels of aggressiveness and presence in the mycobiota. Dual cultivation was carried out at potato-glucose agar (PGA), comparing with single fungal cultivation. The mutual influence of fungi during joint cultivation was established. *Alternaria arborescens*, which dominated in the mycobiota of wheat seeds from northeastern Ukraine, suppressed the development of only *Penicillium*. *Nigrospora oryzae, Bipolaris sorokiniana,* and *Phoma* developed faster than *A. arborescens*. *Fusarium poae,* and *F. sporotrichioides* competed for agar medium with *N. oryzae*. Known bioagents from wheat seeds showed unexpectedly low results. *Trichothecium roseum* formed a rejection zone during co-cultivation with *F. graminearum*. *Trichoderma* sp. Max18 (resistant to fludioxonil) on the 7th day inhibited the development of *Penicillium, F. graminearum,* and *A. arborescens* by 55, 48 and 26%, respectively. *N. oryzae* developed faster than the mycoparasitic fungus, but the latter began to parasitize it only from the 13th day.

Keywords: bioagents, fungal co-cultivation, endophytes, mycobiota, plant protection

Introduction

Of the world's population 40% consumes wheat, which provides 12–13.5% protein and significant caloric content (Giraldo *et al.* 2019). Wheat is not grown under sterile conditions, so it constantly interacts with various microorganisms. Earlier researchers paid more attention to the study of phytopathogenic species, while now many studies are devoted to endophytes (Latz *et al.* 2021; Noel *et al.* 2022). This group of microorganisms enters plants as pathogens (Ahlawat *et al.* 2022). The endophytic mycobiota of wheat is quite diverse, and it is determined by various factors. From 1750 samples of various organs of wheat, 722 fungal isolates were isolated, of which 30 genera were identified (Larran *et al.* 2007). Among wheat endophytes, species have been identified to control pathogens of this crop (FHB pathogens) (Noel *et al.* 2022). They are the most promising for the production of biofungicides, as they have similar ecological niches with phytopathogens and more actively produce secondary metabolites than soil species (Ulloa-Ogaz *et al.* 2015). Endophytes from wheat improve plant growth and increase resistance to abiotic stresses (Ripa *et al.* 2019).

The study of influencing factors on the formation of the endophytic microbiome of wheat showed that the plant organ had the greatest influence on this factor. The fungal community from the root was the most diverse, compared to the seeds and leaves. Moreover, most representatives were unique for each organ, although *Fusarium, Alternaria, Neoascochyta* and *Acremonium* sp. were common in all tissues. (Latz *et al.* 2021). Dissection of mycobiota from leaves, stems, scales and grains showed greater colonization by fungi of the first organ of the plant (Larran *et al.* 2007).

According to our analysis of modern literature, it was established that in the 21st century the mycobiota of wheat seeds includes the following genera: *Acremonium* sp. *Alternaria* sp. (*Ulocladium* sp. belongs 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., *Rhizopus* sp., *Rhizoctonia* sp., *Stemphylium* sp., *Trichoderma* sp., *Trichothecium* sp., and *Verticillium* sp. The mycobiome composition of wheat seeds depends on the research method: 128 species from 81 genera were determined by amplicons sequencing and 52 species from 23 genera by the biological method (Ren *et al.* 2023). The formation of seed mycobiota is influenced by environmental conditions, especially for the accumulation of genera: *Alternaria, Epicoccum*, *Monographella* and *Mycosphaerella* sp. They did not colonize the seeds for growing the plants indoors (Latz *et al.* 2021). Seed endophytes can affect the root mycobiota (Ridout *et al.* 2019) and can be transmitted vertically to wheat seedlings (Huang *et al.* 2016).

The main direction of modern research is the understanding of the peculiarities of the formation of wheat endophyte communities with the isolation of useful representatives. The influential factors of environmental conditions, crop genotypes, organs and tissues, stages of wheat development on fungal complexes have already been studied (Błaszczyk *et al.* 2021). It is unclear how endophytic fungi interact with each other. Scientists suggest the possibility that slow-growing fungi on nutrient media may be undercounted due to their displacement by fast-growing species (Błaszczyk *et al.* 2021). Therefore, the purpose of our study was to study the nature of the interaction of endophytic fungal species of winter wheat seeds with different representations in the mycobiota and growth rate.

Materials and Methods

Fungi were isolated from the seeds of winter wheat from different varieties during the analysis of seed mycobiota of the 2018 and 2019 harvests (northeastern Ukraine). Fungi were identified by colony morphology and anamorphs (Watanabe 2002; Leslie and Summerell 2006).

Co-cultivation of mycobiota fungi of winter wheat seeds was carried out on PGA medium, grown in a thermostat a t22–25°C. Seven-day-old cultures were sown in Petri dishes at a distance of 40–45 mm. As a control, there were variants of single cultivation of the studied fungi. The nature of the relationship was followed in terms that would clearly demonstrate the peculiarities of fungal growth. The diameter of colonies of species and genera was measured perpendicularly. The percentage of inhibition was calculated using the formula: I (%) = 100 [(GC-GT)/GC], where GC (growth control) represents the mean diameter of fungi grown in PDA, and GT (growth in a dual culture) (Ebadzadsahrai *et al.* 2020).

In 2018, four experiments were conducted: in the first experiment, the peculiarities of the simultaneous cultivation of *Nigrospora oryzae* (Berkeley et Broome) Petch. and *Alternaria arborescens* E.G. Simmons were investigated for 7 days, in the second – *Phoma* and *A. arborescens* for 13 days (three-fold repetition), in the third – *A. arborescens* and *Bipolaris sorokiniana* (Sacc.) Shoemaker for 7 days (four-fold repetition), in the fourth – *A. arborescens* and *Penicillium* in three repetitions for 7 days. In 2019, the nature of the interaction between *N. oryzae* and *Fusarium poae* (Peck.) Wollenw., *F. sporotrichioides* Sherb. was studied in three repetitions. Research on the interaction of endophytic fungi with known bioagents was conducted in 2018. The latter also sprouted from seeds. The nature of the interaction of mycobiota components with *Trichoderma* sp. was determined according to the recommendations of Poliksenova *et al.* (2004).

Statistical analysis of the results was performed by the method of one-way Annova in Statistica 10, calculating the LSD_{05} by Dospekhov (2013).

Results

Co-cultivation of fungi aimed to clarify the nature of seed endophyte interactions. We also wanted to determine the peculiarities of the release of seed fungi into the environment, namely if they interfere with each other, or not.

Having received data on the highest growth rate of *N. oryzae*, we decided to investigate its effect on the dominant species of mycoflora of winter wheat seeds – *A. arborescens*, by carrying out their simultaneous cultivation on the medium (Table 1).

Nigrospora oryzae actively developed under joint cultivation with *A. arborescens*, apparently without the presence of another fungus. It grew on a colony of *Alternaria* fungus already on the 6th day and occupied the entire surface of the environment without any obstacles. Colony development of *A. arborescens* was inhibited. On the 6th day, the fungus completely stopped its development.

Co-cultivation of *Phoma* sp. and *A. arborescens* showed a completely different coexistence of fungi. The first species developed faster than the second. On the 7th day, a meeting of their colonies took place in three repetitions (Table 2).

Table 1. Co-cultivation of *Alternaria arborescens* and *Nigrospora oryzae* (2018)

Species of fungi	Colony diameter [mm]		
	the 3rd day	the 6th day	the 7th day
N. oryzae	46 × 43	90 × 90	90 × 90
A. arborescens	17 × 14	35 × 30	40 × 38
A. arborescens+ *N. oryzae*	14 × 12 + 46 × 46	20 × 23 + It grew on *A. arborescens* and occupied the entire dish	20 × 23 + It grew on *A. arborescens* and occupied the entire dish
Inhibition of *A. arborescens* [%]	18.8	33.3	43.6

Table 2. Co-cultivation of *Phoma* sp. and *Alternaria arborescens* (2018)

Species of fungi	Colony diameter [mm]				
	the 3rd day	the 6th day	the 7th day	the 8th day	the 13th day
A. arborescens	12.8 × 10	28.5 × 25.3	37.8 × 26.3	42.8 × 31	48.8 × 49.8
Phoma sp.	17 × 17	41.5 × 34.5	50 × 41	60.8 × 43	81.3 × 48.5
LSD_{05}	5.6	10.6	14.4	7.5	7.7

After meeting, *Phoma* sp. began to occupy the territory that *A. arborescens* did not have time to occupy. The second species turned out to be depressed. It lagged behind in growth from its single cultivation, although its development continued. But *Phoma* sp. quickly covered it and occupied the entire surface of the medium in the Petri dishes. This happened on the 13th day of joint cultivation of fungi. *A. arborescens* also affected the colony development of *Phoma* sp. If on the 3rd day of cultivation, *Phoma* colony had the same diameter, then starting from the 6th day, the diameter of the colony from the side of *Alternaria* became smaller even before they met.

The study of the joint cultivation *of A. arborescens* and *B. sorokiniana* demonstrated a higher growth rate of the second species (Fig. 1)

Bipolaris sorokiniana was more aggressive, occupying a larger area of the environment with its colony. *A. arborescens* lagged behind in growth already on the 2nd day of cultivation. With each recording date, the difference in the diameter of the two colonies gradually increased.

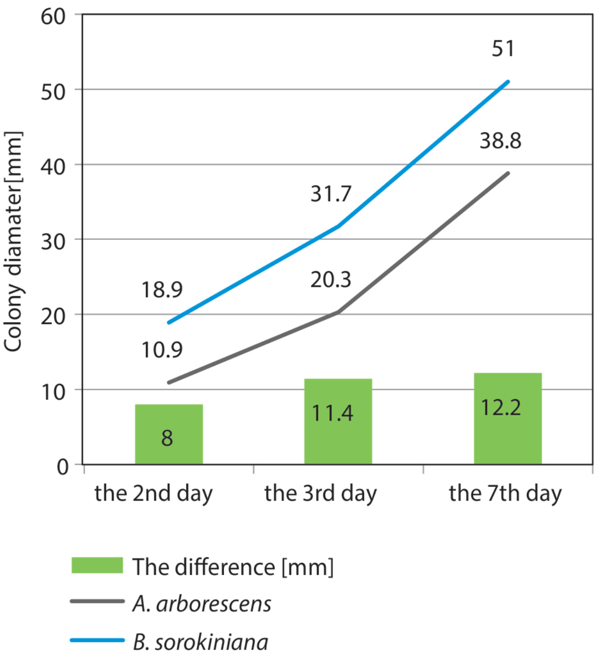

Fig. 1. Joint cultivation of *Alternaria arborescens* and *Bipolaris sorokiniana* (2018) (LSD_{05}7 = 6)

Co-cultivation of *A. arborescens* with *Penicillium* gave results that were opposite to the three previous experiments: the first species had a higher rate of development (Table 3).

Alternaria arborescens from the 2nd day had a larger colony diameter than *Penicillium*. Gradually, the difference between the diameter of the colonies increased. But unilateral development of *A. arborescens* colony was noted. The side of the colony near *Penicillium* had a smaller diameter. That is, *Penicillium* influenced the development of *A. arborescens* .

In 2019, we wanted to determine how *Fusarium* fungi behave under joint cultivation with the most aggressive species of wheat seeds mycobiota – *N. oryzae* (Table 4).

During the joint cultivation of *Fusarium* sp. with *N. oryzae*, an insignificant inhibition of fungi was noted, compared to their individual cultivation. *N. oryzae* developed faster and therefore occupied a larger area of the medium. It did not grow on *Fusarium* sp. as it did on *A. arborescens*. There was only competition for the medium. *N. oryzae* overthrew the *Fusarium* fungus colony on the 5th day of observation.

Table 3. Co-cultivation of *Alternaria arborescens* and *Penicillium* (2018)

Day	*A. arborescens*		*Penicillium*		LSD_{05}
	width and length [mm]	average diameter [mm]	width and length [mm]	average diameter [mm]	
The 2nd	12.7 × 9.7	11.2	7 × 8.7	7.9	3.2
The 3rd	21.7 × 15.3	18.5	10.7 × 11	10.9	5.0
The 7th	44.3 × 40.3	42.3	19.3 × 19.7	19.5	6.3

Table 4. Joint cultivation of *Nigrospora oryzae* and *Fusarium* sp. (2019)

Species of fungi	Colony diameter on the 5th day [mm]		LSD_{05}
	width and length	average diameter	
F. poae	45 × 45	45	
F. poae+ N. oryzae	45.7 × 44 + 90 × 46	44.9 + 68	6.8
LSD_{05} (*F. poae*)		not significantly	
F. sporotrichioides	44 × 37.7	40.9	
F. sporotrichioides + N. oryzae	39.3 × 37.7 + 90 × 51	38.5 + 70.5	8.8
LSD_{05} (*F. sporotrichioides*)		not significantly	

The next step in studying the interaction of fungi during their co-cultivation was to investigate the effect of known biological agents, which were also isolated from winter wheat seeds.

Since *Trichothecium roseum* (Pers.) Link is known as an active producer of the antibiotic trichomycin, we decided to investigate its effect on one of the most dangerous representatives of the mycoflora of the seeds – *F. graminearum* (repetition three times) (Table 5).

At first, fungi grew to meet each other. *T. roseum* developed as under single cultivation on the 5th and 6th days. Starting from the 7th day of cultivation, a decrease in growth indicators of this fungus was noted. The development of *F. graminearum* was slower from the 5th day than when grown alone. It developed better than *T. roseum*. A zone of "rejection" was formed between fungi, and they actively filled the territory of the medium that they occupied. This zone was formed as a result of the production of an antibiotic by *T. roseum*.

Trichoderma sp. was isolated from the seed after its treatment with Maxim 0.25 FS (*Trichoderma* sp. Max18 isolate). This fungus was also resistant to the fungicide, or rather to its active ingredient – fludioxonil. We investigated its effect on mycobiota fungi of wheat seeds in 2018 (Table 6).

Fungi of the genus *Penicillium* formed several colonies on the medium. Their number increased from four (on the 4th day of cultivation) to 10 (on the 7th day of cultivation) during single cultivation. During the simultaneous cultivation of fungi, suppression of the growth of colonies and their number was noted already on the 4th day. On the 7th day, seven of them were formed. The percentage of *Penicillium* inhibition ranged from 41 to 55%. The highest rate was noted on the 7th day of cultivation, when these fungi stopped their growth under the influence of the parasite. But around large *Penicillium* colonies a rejection zone was formed. *Trichoderma* had the worst sporulation compared to co-cultivation with other fungi.

On the 6th day, *Trichoderma* grew on *F. graminearum*, which stopped developing. Its colonies began to actively form on the phytopathogenic fungus. On the 12th day, the *Fusarium* colony changed its

Table 5. Co-cultivation of *Fusarium graminearum* and *Trichothecium roseum* (2018)

Day	*F. graminearum*		*T. roseum*		LSD_{05}
	width and length [mm]	average diameter [mm]	width and length [mm]	average diameter [mm]	
The 5th	26 × 27.7	26.9	24.7 × 24	24.4	not significantly
The 6th	35 × 33	34	34.7 × 32.7	33.7	not significantly
The 7th	42.7 × 42.7	42.7	40 × 40.6	40.3	2.4
The 12th	75 × 45.3	60.2	63.7 × 41.7	52.7	5.6
The 15th	78.3 × 48.3	63.3	69.7 × 41.7	55.7	5.4

Table 6. Inhibition of fungal growth from winter wheat seeds by *Trichoderma* sp. Max18 (2018)

Genus/species	Inhibition on the 7th day [%]	Features of growth endophytic fungi	Peculiarities of parasitism of *Trichoderma* sp.
Penicillium	55	stopped development on the 7th day	rejection zone
F. graminearum	48	stopped development on the 6th day	on the 6th day, it grew into fungus, changed the color of its colony
A. arborescens	26 (on the 9th day – 48)	stopped development on the 8th day	on the 9th day, it grew into a colony and changed its color.
N. oryzae	–	filled the Petri dish on the 6th day	on the 13th day, it began to form colonies and actively grow on the surface of the fungus

color to light brown. On the 4th day, *A. arborescens*, which was grown with Trichoderma sp., developed faster. On the 7th day, 26% inhibition of *Alternaria* fungus was noted compared to its single cultivation. On the 8th day, the growth of the *A. arborescens* colony was stopped. It began to change its color, which indicates the destruction of the pigment. On the 9th day, the colony became light brown, and the mycelium of *Trichoderma* sp. grew on an *Alternaria* fungus. In relation to *F. graminearum* and A. arborescens, the presence of fungistatic alimentary unilateral antagonism by *Trichoderma* was noted. *N. oryzae* grew faster on the medium than the Max18 isolate. On the 6th day, it grew a fungus from the genus *Trichoderma* and stopped its development. Monitoring was suspended. But on the 13th day, it was noted that Max18 began to actively parasitize *N. oryzae.*

Discussion

Among the endophytes, *Alternaria* sp. is dominant in seeds from different places of wheat cultivation (Ramires *et al.* 2018; Turzhanova *et al.* 2020; Shabana *et al.* 2021). Ukraine is no exception (Ostrovsky *et al.* 2018; Golosna 2021). In northeastern Ukraine, the species *A. arborescens* turned out to be the most common (Rozhkova *et al.* 2021), so it was included in the study in order to understand the reason for its dominance. It suffered a negative effect from other fungi due to their joint cultivation. *N. oryzae, Phoma* sp. and *B. sorokiniana* suppressed the development of *A. arborescens.* But it had a negative effect on other fungi, especially *Penicillium*, in addition to *N. oryzae*. This species had the highest growth rate according to earlier observations (Rozhkova *et al.* 2022). Despite the suppression by other fungi, the insignificant growth rate on the medium, *A. arborescens* occupied a dominant position in the seed mycobiota. Co-cultivation of fungi allows, not only the study of the interaction of fungi, but also to discover new microbial metabolites. Co-cultivation of *N. oryzae* with *Beauveria bassiana* led to suppression of the latter and the discovery of new azaphilones. (Zhang *et al.* 2018). *Fusarium* sp. developed faster than *Alternaria* sp., but was inferior to *N. oryzae* (Rozhkova *et al.* 2022). Therefore, we decided to test the interaction of *Fusarium* sp. from *N. oryzae*. In addition, it is known that *Nigrospora sphaerica* has antagonistic potential against *Fusarium oxysporum* f. with cubense (Foc) (Shara *et al.* 2023).

T. roseum was chosen for the study because it is a known bioagent in plant protection. In Ukraine, a preparation based on this type of antibiotic (trichothecin) was actively used under indoor conditions (Markov 2014). The wheat seed isolate did not show antifungal activity against *F. graminearum*, although *T. roseum* is known to be effective against rust (Kumar and Jha 2002), and powdery mildew (Zhu *et al.* 2022).

Trichoderma sp. Max18 had a slow antifungal effect despite inhibiting the growth of the tested fungi. It did not stop the development of *N. oryzae*, but later used it for nutrition. Strains of *Trichoderma* sp., which are obtained from the soil, are more effective than ours. *Trichoderma asperellum* GDFS1009 showed 60% inhibition of mycelial growth of *F. graminearum*, the causative agent of corn stalk rot (He *et al.* 2019). *Trichoderma viride* F-100076 significantly inhibited the development of *Alternaria radicina*, *Fusarium oxysporum*, *F. solani, F. oxysporum* var. orthoceras, *F. moniliforme* var. lactis, etc., forming active sporulation on the studied species under joint cultivation (Kopilov *et al.* 2020).

Conclusions

In most cases co-cultivation demonstrated a mutual influence of endophytic fungi from wheat seeds. Most *N. oryzae* suppressed the development of *A. arborescens* colonies, when it grew on the Alternaria fungus on the 6th day of cultivation. *N. oryzae* also had the least influence on the development of colonies of *Fusarium* sp. (*F. poae* and *F. sporotrichioides*). *B. sorokiniana* and *Phoma* fungi also suppressed *A. arborescens*. *Penicillium* was negatively affected by the *Alternaria* species.

F. graminearum developed better than *T. roseum* in dual culture. *Trichoderma*, which was isolated from the seeds after it was treated with Maxim 0.25 FS, proved to be the most effective against *Penicillium*, stopping the development of its colonies on the 7th day. The development of *A. arborescens* was stopped on the 8th day, and *F. graminearum* – on the 6th. The effect on *N. oryzae* was manifested only after filling the Petri dish with this fungus. *Trichoderma* actively grew on *N. oryzae* on the 13th day. The parasitic isolate changed the color of the fungal colonies.

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