Species diversity of *Fusarim* fungi on grain crops in the conditions of the foothill zone of the Central Caucasus

I R Manukyan[1*], *R M* Khatsaeva[2], *S G* Kosyrev[1], and *M X* Al Azawi Nagam[3]

[1]Nort Caucasian Researsh Institute of Mountain and Submontain Agriculture branch of Vladikavkaz Scientific Center of Russian Academy of Sciences, 1, settlement Mikhailovskoe Williams str., Vladikavkaz, Russian Federation
[2]Institute of Ecology and Evolution named after A.N. Severtsov RAS, 119570, 33, Leninsky av., Moscow, Russian Federation
[3]University of Baghdad, Faculty of Agricultural Engineering, Baghdad, Iraq

Abstract. The article presents the results of studies of the species composition of phytopathogens of winter soft wheat grain affected by ear fusarium. For the first time, studies were conducted using a complex of electron microscopic, molecular genetics, and mycological methods. The species F. graminearum dominated in all samples (frequency of occurrence 42.8%). The species F. avenaceum (23.3%), F. sporotrichioides and F. equiseti (8.8% each) were noted with high frequency. Next in descending order of frequency of occurrence: F. oxysporum, F. boothii, F. poae, F. proliferatum, F. accumatum, F. incarnatum, F. vorosii, F. tricinctum, F. cerealis, F. sambuticum. For the first time, 7 species were found for the plain zone of the Pre-Caucasus: F. equiseti, F. boothii, F. vorosii, F. cerealis, F. sporotrichioides, F. oxysporum, F. incarnatum.

1 Introduction

Fusarium ear blight (FHB) is a common disease of cereal crops worldwide and is among the top five most destructive fungal pathogens affecting agroecosystems. Globally, FHB is considered as the most dangerous and damaging disease of wheat, which causes high economic losses, especially in humid climate regions [1-2, 4-5]. The incidence of epidemics of *Fusarium* ear blight has increased significantly over the last decade. Climate change as well as changes in cropping systems have contributed to the gradual spread of FHB across regions where it is a major threat to cereal crop production [3, 6, 9]. The species composition of *Fusarium* ear blight pathogens is determined by the climatic conditions of the region and the set of cereal crop varieties [10-11]. Changes in climatic conditions contribute to changes in the species diversity of the pathogenic complex [15].

A significant contribution to the study of the biology of fungi of the genus *Fusarium* and species identification belongs to molecular research methods. For example, the use of DNA markers allowed us to identify nine phylogenetic species within the species of *F.*

* Corresponding author: r.khatsaeva@mail.ru

graminearum. Phylogenetic analysis based on tefla gene sequences allowed to identify two clusters (North European and South European) within the *F. equiseti* species. New taxonomic groups were also discovered within the species complexes of *F. fujikuroi* and *F. solani* [8, 12-14].

The nucleotide fragments of the genes of translation elongation factor 1-α (*tef1*), β-tubulin (*tub2*), and histone H3 are most often analyzed in phylogenetic studies of fungi of the genus *Fusarium* [16].

The molecular genetic approach made it possible to assess the relationship between species, which was not obvious when analyzing only morphological characters. As an example, the species *F. tricinctum*, traditionally considered as closely related to *F. sporotrichioides* and *F. poae* and attributed to the section *Sporotiichiella* [13]. At the same time, when comparing species by the profile of toxic metabolites, such as enniatins and moniliformin, it was found that *F. tricinctum* differs from *F. sporotrichioides* and *F. poae*, which secrete mainly mycotoxins of the trichothecene group. Phylogenetic analysis confirmed the close relationship of *F. tricinctum*, *F. avenaceum* and *F. acuminatum* [14]. Until recently, *F. graminearum* was considered as a single polymorphic species. Subsequently, multilocus molecular analysis revealed phylogenetically different lineages that received the species rank: *F. boothii* and *F. vorosii* [20-21].

The aim of the research was a comprehensive study of the species composition of the causative agents of fusariosis of winter soft wheat ear. Determination of dominant species and frequency of occurrence of fungi of the genus *Fusarium* on ear and grain of winter wheat and triticale in the conditions of the foothill zone of the Central Caucasus.

2 Materials and methods

The object of research is the grain of winter soft wheat and triticale healthy and with symptoms of fusariosis of ear. For comparison we took medium resistant winter wheat variety Dorota (France), medium susceptible variety Batko (Russia) and resistant winter triticale variety Gor (Russia). The isolation of fungi of the genus *Fusarium* and species identification were carried out according to cultural-morphological and molecular-genetic characteristics in the laboratory of mycology of FGBU "VNIIKR". Isolates (180) were isolated in pure culture under sterile conditions according to the generally accepted methodology. The fungi were cultured for 14 days on 2% potato-glucose agar at 25^{0}°C. Olympus Bx43F microscope was used, Quick-photo MICRO 3.2 software was used for morphometry. To confirm species affiliation, classical PCR with universal primers Tub 2F (GTBCACCTYCARACCGGYCARTG)/Tub 4R (CCRGAYTGRCCRAARARAAG TTGTC) with further sequencing of nucleotide sequences according to the Sanger method on a 3500 Applied Biosystems genetic analyzer [17-18] was performed.

For sequencing of taxonomically informative loci and identification of Fusarium fungi, the β-tubulin gene region was amplified. Sequences of β-tubulin genes obtained from GenBank (NCBI) and FUSARIUM-ID databases were used as references [1, 6, 10]. Electron microscopic studies were performed on the equipment of the Center for Collective Use "Instrumental Methods in Ecology" using the latest scanning electron microscopy (SEM) methods. Samples were fixed in 10% buffered formalin, washed first in flowing and then in distilled water, passed through alcohols of increasing concentration (from 30 to 100%) and acetone, dried in critical point on Hitachi Critical Point Dryer HCP-1 ("Hitachi", Japan). Then, the dried samples were mounted with double-sided carbon tape on special slides and sputtered with gold in vacuum on a Q150R ES Plus machine (QuorumTechnologiesLtd, UK). Then, to create a conductive surface, the prepared samples were examined on a MIRA 3 LMH scanning electron microscope (TESCAN, Czech Republic) [7].

3 Results and Discussion

Climatic features of the foothill zone of the Central Caucasus are unstable moisture content and uneven precipitation during the growing season. The maximum precipitation (152-202% of the norm) falls during earing and flowering (May-June), the most vulnerable period for infection by Fusarium ear blight. Due to the peculiarities of weather conditions, fusarium epiphytotics occur almost annually. In the years of epiphytotics, all varieties of both foreign and Russian selection are affected to varying degrees. The variety Sumai aut (China) and its hybrids with red coloration of ear and grain are more resistant [9].

The previous study of the species composition of fungi of the genus *Fusarium* was conducted in the early 2000s by the staff of the laboratory of mycology and phytopathology of the All-Russian Research Institute of Plant Protection. Seven species were identified, the dominant species were *F. graminearum* and *F. poae* (Table 1) [8].

Table 1. Species composition of *Fusarium* ear blight pathogens of winter soft wheat.

No	Species of fungi of the genus *Fusarium*	Frequency of occurrence, %
1	*F. graminearum*	82
2	*F. poae*	11
3	*F. avenacium*	2
4	*F. tricinctum*	1
5	*F. culmorum*	1
6	*F. virticilioides*	2
7	*F. accuminatum*	1

Studies conducted by us in 2022 expanded the ideas about the diversity of *Fusarium* fungi in the ear and grain pathocomplex. Species affiliation was determined by electron microscopic, culture-morphological and molecular-genetic methods. As a result, 14 species of fungi of the genus *Fusarium* were identified (Table 2).

Table 2. Species composition of *Fusarium* pathogens of winter soft wheat ear.

No	Species of fungi of the genus *Fusarium*	Number of isolates / frequencies of occurrence, %
1	*F. graminearum*	77/ 42.8
2	*F. avenacium*	42/23.3
3	*F. equiseti*	16/8.8
4	*F. sporotrichioides*	16/8.8
5	*F. oxysporum*	7/3.9
6	*F. boothii*	5/2.8
7	*F. poae*	3/1.7
8	*F. proliferatum*	3/1.7
9	*F. accuminatum*	3/1.7
10	*F. incarnatum*	2/1.1
11	*F. vorosii*	2/1.1
12	*F. tricinctum*	2/1.1
13	*F. cerealis*	1/0.6
14	*F. sambuticum*	1/0.6

An increase in diversity was found due to the following species: F. equiseti, F. sporotrichioides, F. oxysporum, F. boothii, F. proliferatum, F. incarnatum, F. vorosii, F. sambuticum.

In all grain samples, *F. graminearum* dominated (frequency of occurrence 42.8%). Species of *F. avenaceum* (23.3%), *F. sporotrichioides* and *F. equiseti* (8.8% each) were recorded with high frequency. Further, in decreasing order of frequency of occurrence: *F.*

oxysporum, F. boothii, F. poae, F. proliferatum, F. accuminatum, F. incarnatum, F. vorosii, F. tricinctum, *F. cerealis, F. sambuticum.* A rather high frequency of occurrence (8.8%) was noted for *F. equiseti*, which is more characteristic of Siberia and the Far East [2].

The established species specificity of fusarium fungi isolated from grain with and without symptoms of FHB confirms the fact that infection of ear and grain occurs continuously from the flowering phase to harvesting. During the initial periods, the ear is infected by more aggressive fungi *F. graminearum*, *F. avenacium*, which have the highest frequency of occurrence. The greatest diversity of species (10 species) was found on the medium resistant variety Batko. Six *Fusarium* species were found in the grain of this variety, but without external symptoms of lesions. The species absent were: *F. oxysporum*, *F. cerealis*, *F. incarnatum*, *F. sambuticum.*

In affected grain of the more resistant variety Dorota 5 species were found, in grain without FHB symptoms *F. graminearum* was not detected, but *F. tricinctum* was found. The most frequently occurring species are: *F. graminearum*, *F. avenacium*, *F. sporotrichioides*, *F. equiseti* (Table 3).

Winter triticale is more resistant to fusarium fungi than winter wheat. Only three species of fusarium fungi were found in diseased triticale grain: *F. graminearum, F. oxysporum, F. equiseti*. Only *F. boothii* species were present in apparently healthy triticale grain.

Table 3. Species composition of fungi of the genus *Fusarium* on winter soft wheat varieties with different resistance levels.

Variety	**Species composition of fungi of the genus *Fusarium***	
	Externally diseased	**Externally healthy**
Batko	*F. graminearum, F.avenacium, F. oxysporum, F.cerealis, F. incarnatum, F. equiseti F. sporotrichioides, F. poae, F.vorosii, F. sambuticum*	*F. graminearum, F.avenacium, F. sporotrichioides, F. equiseti F. accuminatum, F. boothii,*
Dorota	*F. graminearum F. avenacium F. proliferatum, F. equiseti, F. sporotrichioides*	*F. avenacium, F. proliferatum F. equiseti, F. sporotrichioides, F. incarnatum, F. tricinctum,*
Gor	*F. graminearum, F. oxysporum, F. equiseti,*	*F. boothii,*

Judging by the species composition of fusarium fungi, mycotoxins of different types may accumulate in the affected grain: trichothecene toxins of type A (*F. sporotrichioides, F. poae*), trichothecene toxins of type B (*F. graminearum, F. cerealis, F. sambucinum*), enniatine group toxins and moniliformin (*F. avenaceum, F. tricinctum, F. acuminatum*) [1, 6, 18].

The results obtained indicate a community of fusarium fungi permanently present in grain agrocenosis. Species differ among themselves in terms of moisture and temperature requirements, as well as a set of toxic substances of secondary metabolism, providing competitive interactions between species and successful colonization of nutrient substrate. The constant presence in the agrocenosis of a complex of fusarium fungi occupying different ecological niches ensures continuous infection of ear and grain from the flowering phase to harvesting.

Molecular phylogeny methods were used to study 30 isolates isolated from diseased FHB grain of wheat and triticale. As a result of DNA sequencing, the nucleotide sequences of the β-tubulin gene were determined for all the isolates studied. The obtained nucleotide sequences had a size of about 300 bp. After their alignment, the obtained data matrices were used for subsequent phylogenetic analysis.

On the phylogenetic tree constructed on the basis of nucleotide sequences of the β-tubulin gene locus, all isolates were distributed into 16 clades (Figure 1). Five *Fusarium graminearum* isolates F43, F59, F93, F110 were located within the diseased clade formed by the reference isolate MW533966.1 *Fusarium graminearum CBS 136009*. Two isolates F70, F71 were closely related to the reference isolate *MG063785.1 Fusarium graminearum vorosii*.

Isolate F92 formed a clade with two reference strains KJ125832.1 and AF314487.1 *Fusarium cerealis culture collection*.

Isolates F75, F91, F106 formed a single large clade containing the reference sequence HQ141639.1 *Fusarium sporotrichioides NRRL 29978*. In the phylogram, isolate F73 appeared closely related to species of the large clade based on sequences with maximum bootstrap support values with reference type strain AF 404235.1 *Fusarium poae isolate IBT 2963*. Isolates F81, F67, F133 and F117 were included in the *Fusarium equiseti* clade, but within it formed two separate subclades that did not include any reference strains.

Isolates F130, F42, F132 entered a large clade with high bootstrap support values with reference type strains MW533812.1 *Fusarium proliferatum strain LC13672* and *MH398240 Fusarium proliferatum strain NRRL66451*.

Isolates F46, F72, F69 were included in the large *Fusarium oxysporum* clade, but within it formed separate subclades that did not include any reference strain. Isolates F6, F88, F126 were included in the *Fusarium avenaceum* clade with high bootstrap support values with reference type strains *EU490225.1 Fusarium avenaceum isolate ICMP12585*. Isolate F115 was part of a large clade of *Fusarium tricinctum* with high relatedness values with reference type strain *MG990938.1 Fusarium tricinctum isolate SPF00*. Isolates F104, F98 were included in the *Fusarium acuminatum* clade and formed a separate clade that did not include the reference strain.

Scanning micrograph of diseased FHB grain confirmed the existence of a whole complex of microorganisms of different species colonizing and developing on the grain. (Figure 2).

Electron micrographs of fusarium grain showed something that usually remains out of mycologists' field of view. The presented images show folded structures of unknown organisms, most likely fungi, which neither mycologists nor phytopathologists could identify. It is difficult to say what role these organisms play? The question of whether they are phytopathogens, saprotrophs or superparasites remains open (Figure 3).

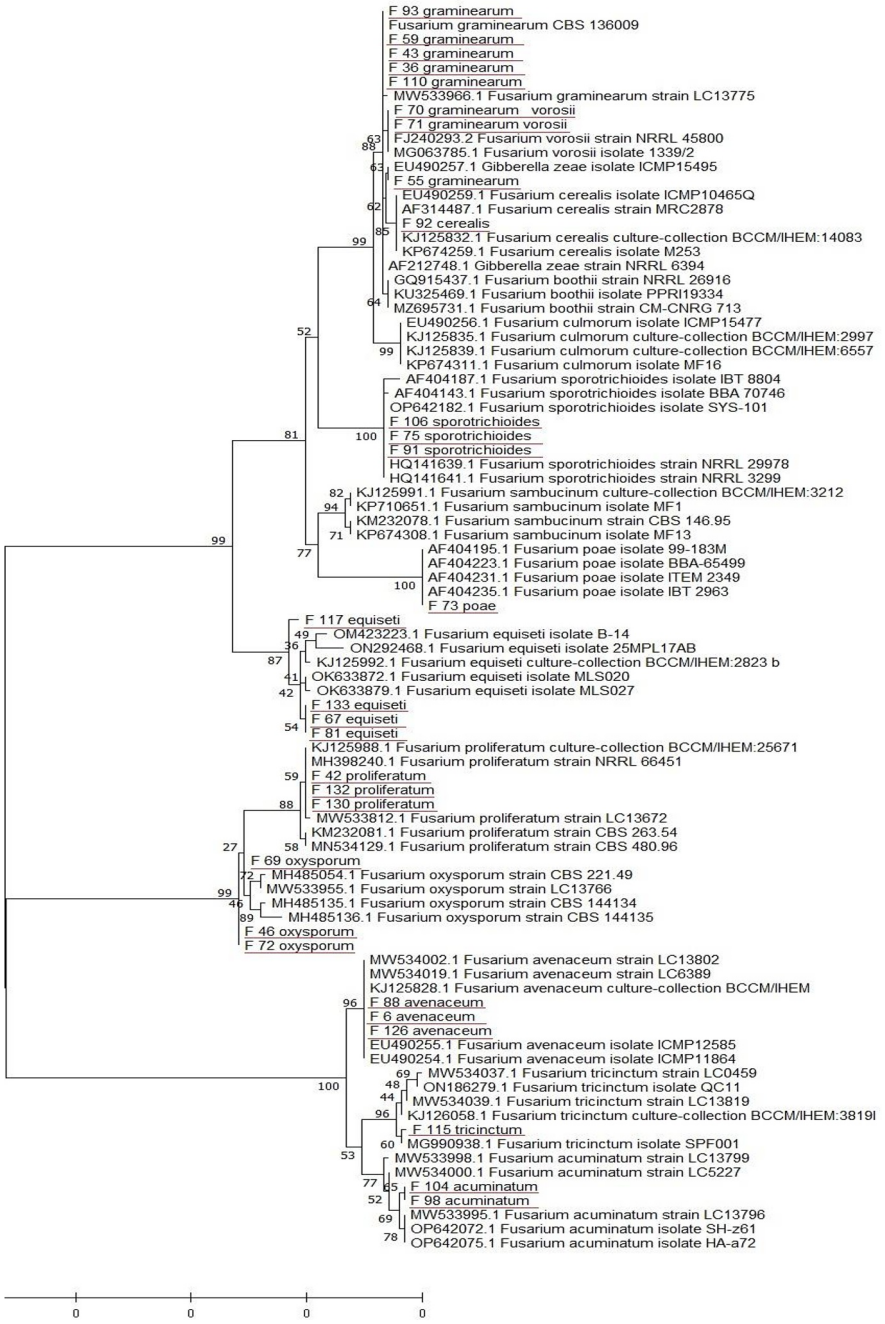

Fig. 1. Phylogenetic tree of *Fusarium* isolates based on sequences of the β-tubulin gene region.

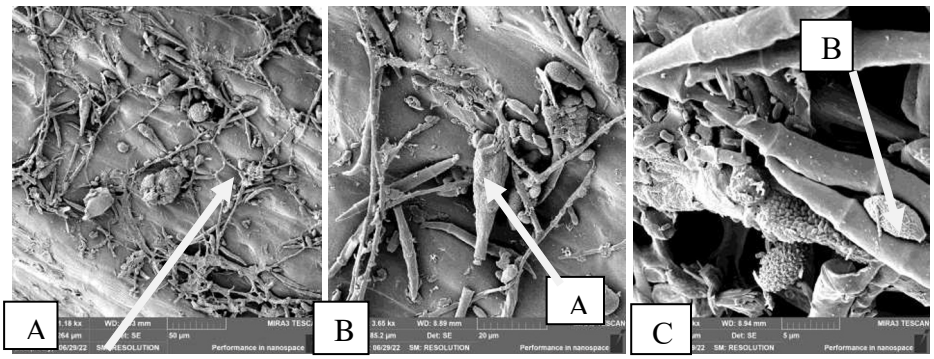

Fig. 2. *Fusarium* grain surfaces: A - *Alternaria sp.* conidia, B - *Fusarium sp.* conidia, C - *Cladosporium sp.* conidia (magnification x 3000-3500).

Fig. 3. Electron microscopic photographs of wheat grain surface with FHB lesions. The arrows indicate unknown fold structures.

4 Conclusion

Thus, the phytopathogenic complex of grain affected by fusariosis is represented by 14 species of fungi from the genus *Fusarium*. The greatest diversity of *Fusarium* species was found on the medium resistant variety Batko (10 species). The most frequently encountered species are: *F. graminearum*, *F. avenacium*, *F. sporotrichioides*, *F. equiseti*. It was revealed that in wheat grain without visible symptoms, there is a latent infection with Fusarium ear blight represented by 5-6 species. For the first time 7 species were identified for the foothill zone of the Central Caucasus: *F. equiseti*, *F. boothii*, *F. vorosii*, *F. cerealis*, *F. sporotrichioides*, *F. oxysporum*, *F. incarnatum*.

The use of modern methods in phytopathological analysis showed the biological diversity of species occurring in the affected ear and grain. In addition to fusarium fungi, associated phytopathogens were found: *Helmintosporium*, *Alternaria*, *Cladosporium*, *Epicoccum*, as well as biological entities whose systematic position and functional significance are not yet known.

References

1. T.Yu. Gagkaeva, O.P. Gavrilova, M.M. Levitin, Biodiversity and distribution of the main toxigenic Fusarium fungi, Biosfera, **6**, **31**, 36-45 (2014) DOI:10.24855/biosfera.v6i1.202
2. G.N. Buchneva, I.V. Gusev, O.I. Korabelskaya, N.N. Dubrovskaya, V.V. Chekmarev, Varietal composition and frequency of Fusarium presence on winter wheat varieties in the Tambov region, Grain economy of Russia, **2**, **62**, 74-76 (2019) DOI 10.31367/2079-8725-2019-62-2-74-76
3. M.M. Levitin, V.G. Dzhavakhiya, Toxigenic fungi and food security issues (review), Achievements of Science and Technology of AIC, **34**, **12**, 5–11 (2020) DOI: 10.24411/0235-2451-2020-11201
4. I.R. Manukyan, Phytopathogens of winter wheat in North Ossetia, Plant Protection and Quarantine, **1**, 32-33 (2003)
5. I.R. Manukyan, M.A. Basieva, E.S. Miroshnikova, V.B. Abiev, Assessment of ecological plasticity of winter wheat varieties in the conditions of a foothill zone of the Central Caucasus, Agrarian Bulletin of Ural, **4**, **183**, 20-26 (2019) DOI: 10.32417/article_5cf94f63b4d0f7.46300158
6. A.A. Stakheev, L.V. Samokhvalova, D.Yu. Ryazantsev, S.K. Zavriev, Molecular genetic approaches for investigation of taxonomy and specific identification of toxin-producing Fusarium species: achievements and problems (review), Agricultural Biology, **51**, **3**, 275-284 (2016) doi: 10.15389/agrobiology.2016.3.275rus
7. R.M. Khatsaeva, Morphofunctional study of the digestive organs of Bovids (Bovidae), Methodical recommendations. Moscow: Partnership of scientific publications KMK, ISBN 978-5-6040241-6-4, 96 (2017)
8. A.A. Shvartsev, M.L. Koroleva, S.A. Blinova, V.S. Rubets, V.V. Pylnev, Molecular genetic analysis for Fusarium species diversity in the winter triticale (×Triticosecale Wittm. Ex A. Camus), Bulletin of Perm University, Biology, **1**, 65–75 (2023) doi.org/10.17072/1994-9952-2023-1-65-75
9. M. Kebede, G. Adugna, B. Hundie, Status of Fusarium head blight on wheat fields in Southwestern Ethiopia, Italian Journal of Mycology, **50**, **1**, 117–130 (2021) doi.org/10.6092/issn.2531-7342/13320

10. T. Abdissa, B. Bekele, T. Selvaraj, Evaluation of Response of Wheat (Triticum spp) genotypes against Wheat Fusarium Head Blight in Ethiopia, Advances in Crop Science and Technology, **10**, **4**, 505 (2022) DOI: 10.4172/2329-8863.1000505
11. E. Alisaac, A.K. Mahlein, Fusarium head blight on wheat: Biology, modern detection and diagnosis and integrated disease management, Toxins,.**15**, **3**, 192 (2023) DOI:10.3390/toxins15030192
12. N.S. Chandra, E.G. Wulff, A.C. Udayashankar, B.P. Nandini, S.R. Niranjana, C.N. Mortensen, H.S. Prakash, Prospects of molecular markers in Fusarium species diversity, Applied Microbiology Biotechnology, **90**, **5**, 1625-39 (2011) doi: 10.1007/s00253-011-3209-3
13. K. O'Donnell, T.J. Ward, D. Aberra, Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the Fusarium graminearum species complex from Ethiopia, Fungal Genet. Biol., **45**, 1514-1522 (2008) doi: 10.1016 / j.fgb.2008.09.002
14. K. O'Donnell, T.J. Ward, D.M. Geiser, Genealogical concordance between mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the Fusarium graminearum clade, Fungal Genet. Biol., **41**, 600-623 (2004) doi: 10.1016/j.fgb.2004.03.003
15. T. Birr, M., Hasler J.A. Verreet, H. Klink, Composition and Predominance of Fusarium Species Causing Fusarium Head Blight in Winter Wheat Grain Depending on Cultivar Susceptibility and Meteorological Factors, Microorganisms, **8**, **4**, 617 (2020) doi: 10.3390/microorganisms8040617
16. Marin P., Moretti A., Ritieni A., Jurado A.M., Vazquez C., Gonzalez M.T. Jaen Phylogenetic analyses and toxigenic profiles of Fusarium equiseti and Fusarium acuminatum from cereals from Southern Europe, Food Microbiolody, 2012, - No.31.- P. 229-237. doi:10.1016/j.fm.2012.03.014
17. F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. USA, **4**, **12**, 5463-5467 (1977) doi: 10.1073/pnas.74.12.5463
18. F. Sanger, A.R. Coulson, A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase, Journal of Molecular Biology, **94**, 441-448 (1975) doi: 10.1016/0022-2836(75)90213-2
19. L.R. Gale, S.A. Harrison, T.J. Ward, Nivalenol-type populations of Fusarium graminearum and F. asiaticum are prevalent on wheat in southern Louisiana, Phytopathology, **101**, 124-134 (2011) DOI: 10.1094/ФИТО-03-10-0067
20. S.N. Wegulo, E. Valverde-Bogantes, C. Dolanos-Carriel, H. Hallen-Adams, A. Bianchini, N. McMaster, D.G. Schmale, First Report of Fusarium boothii Causing Head Blight of Wheat in the United States, Plant Disease, **102**, **12**, 2646-2667 (2018) DOI:10.1094/PDIS-04-18-0696-PDN
21. S.G. Leyva-Mir, E. García-León, M. Camacho-Tapia, H.E. Villasenor-Mir, K.Y. Leyva-Madrigal, G.A. Mora-Romero, J.M. Tovar-Pedraza, Occurrence of the Fusarium incarnatum-equiseti Species Complex Causing Fusarium Head Blight of Wheat in Mexico, Plant Disease, **106**, **10**, 2755 (2022) doi: 10.1094/PDIS-11-21-2467-PDN