

Microbiological quality of grain cultivated in the North Caucasus region in 2019

Tatiana Gagkaeva^{1,*}, Olga Gavrilova¹, Aleksandra Orina¹, Aleksey Burkin², and Kharon Khusaynov³

¹Laboratory of Mycology and Phytopathology, All-Russian Institute of Plant Protection, St. Petersburg, Pushkin 196608, Russia

²Mycotoxicology Laboratory, All-Russia Research Institute of Veterinary Sanitation, Hygiene, and Ecology – Skryabin and Kovalenko Federal Scientific Center All-Russia Research Institute of Experimental Veterinary Medicine, 123022 Moscow, Russia

³Department of Landscape Agriculture, Chechen Research Institute of Agriculture, Grozny, Gikalo village 366021, Russia

Abstract. The microbiological quality of 23 grain samples of wheat and barley harvested in the North Caucasus in 2019 was analysed on the basis of the percentage of grains infected by fungi and the amounts of trichothecene-producing *Fusarium* DNA and *Alternaria* DNA. The mycotoxins produced by these fungi were also determined. *Alternaria* and *Fusarium* fungi were the predominant fungi in the mycobiota of grain, accounting for at 93% and 14% of the observed fungi, respectively. Alternariol produced by *Alternaria* fungi was detected in 65% of samples, and its content (11-675 ppb) was positively correlated with the abundance of fungi of section *Alternaria* in grain. *F. langsethiae* was found in wheat grain from the Chechen Republic for the first time. The T-2 toxin produced by this fungus was found in 25% of samples, and its content in one barley grain reached 650 ppb, which exceeded the permitted level for this mycotoxin. The mycotoxins deoxynivalenol and zearalenone, which are mainly produced by *F. graminearum*, were also identified in 13% of the grain samples. The positive correlation between the amounts of both these mycotoxins and the DNA of *Tri-Fusarium* was established.

1 Introduction

The main export-oriented grain-producing region in Russia is the North Caucasus (Kuban, Don and Stavropol Territories), where 25% of the total annual grain production in Russia takes place. The microbiological quality of grain from this region has been analysed for many years, and the situation of the contamination of grain by toxin-producing fungi is well known [1, 2].

Several surveys of mycotoxin contamination of grain in the Southern European region of Russia have been conducted [3, 4]. In Krasnodar Territory in 2002-2003, grain was mainly contaminated by the T-2 toxin (85.0%) and was less often contaminated by DON (8.3%) and ZEN (0.7%). The T-2 toxin was detected more often in barley grain than in wheat [3].

Unfortunately, the available information about the fungal infection and mycotoxin contamination of cereals cultivated under natural environmental conditions in different Republics of the North Caucasus region is limited.

The urgency of the need for regular control measures for the mycotoxicological quality of produced grain is indicated by the results of the analysis of 37 grain samples of wheat and barley grown in North Ossetia in 2004-2007. High levels of the T-2 toxin (4-223 ppb), DON (40-2510 ppb) and ZEN (50-140 ppb) were

detected in 68%, 59% and 8% of the samples, respectively [3].

The Chechen Republic (or Chechnya) is located in the eastern part of the North Caucasus, which is characterized by considerable variety of climatic conditions, ranging from arid, temperate continental conditions to cold, humid highlands, so the analysis of grain grown in the territory of the Republic is of particular interest.

Cereals occupy approximately 50% of the sown area in Chechnya. According to official information from the Ministry of Agriculture of the Chechen Republic, in 2019, the harvested area of cereals was 110 thousand ha, and the yield of cereal crops averaged 2.43 t/ha, which was significantly lower than in the previous year [5]. This loss was caused by the death of a significant portion of winter and spring crops due to drought beginning in autumn.

The microbiological quality of grain is influenced by many factors and not only by the meteorological conditions prevailing during grain ripening and harvesting. Other determining factors are the applied cultivation technology, seed quality and contamination of obtained crops by fungi.

Temperature and humidity during the vegetation period and during crop storage are key factors affecting the ability of fungi to produce mycotoxins, which are fungal secondary metabolites and have various acute and chronic effects on human and animal health. Mycotoxins

* Corresponding author: t.gagkaeva@mail.ru

represent an important risk, as most consumers worldwide are exposed to persistent levels of multiple mycotoxins in food, especially in cereal products.

The production of mycotoxins by fungi is a pronounced species-specific characteristic; therefore, knowledge about the species composition of fungi allows predicting the spectrum of toxic metabolites contaminating grain.

Widespread groups of *Alternaria* and *Fusarium* fungi infect many crop plants, including cereals. *Alternaria* is a group of ubiquitous fungi that can penetrate grain in the early stages of growth. These fungi located inside the plant tissue are usually not visible and do not cause apparent harm to the host; nevertheless, some *Alternaria* species can produce dangerous mycotoxins [6-9]. Among the toxic secondary metabolites of *Alternaria* fungi, alternariol (AOH) is one of the most important mycotoxins due to its frequent occurrence in grain samples.

The *Alternaria* species that are most actively involved in AOH contamination in cereals and fodder remain unclear [9-13]. AOH is cytotoxic and induces apoptotic cell death through the mitochondrial pathway [14]. However, the amounts of *Alternaria* mycotoxins in cereal grains are not regulated in Russia.

When grain is colonized by toxin-producing *Fusarium* fungi, there is a significant risk of contamination with mycotoxins, and the safety of the consumption grain can consequently be greatly reduced [15]. In the Russian Federation, according to food and feed safety control standards, the maximum permissible limits (MPL) of mycotoxins produced by *Fusarium* fungi have been established for cereal grains and their processed products [16].

The MPL for deoxynivalenol (DON) and zearalenone (ZEN), mainly produced by *F. graminearum* and *F. culmorum*, cannot be higher than 1000 ppb. The MPL of the T-2 toxin, which is one of the most toxic *Fusarium* secondary metabolites to both humans and animals [15, 17] and is produced by *F. sporotrichioides* and *F. langsethiae*, is 100 ppb.

The analysis of the fungal species composition and mycotoxin contamination associated with cereal grain harvested in the territory of the Chechen Republic has not been previously conducted.

The aim of this study was to evaluate microbiological quality and examine the amounts of mycotoxins in the grain of wheat and barley grown in the North Caucasus region in 2019.

2 Materials and Methods

In 2019, twenty wheat and three barley grain samples originating from the Chechen Republic (n=19), Republic of Ingushetia (n=3) and Republic of Dagestan (n=1) were analysed. Eight samples were collected in the dry steppe, 5 in the steppe, 8 in the forest-steppe and two in mountain-forest biomes.

To evaluate the latent infection of grain and for the subsequent identification of the fungal species composition, 100-200 grains of each sample were

surface sterilized with 5% NaOCl and placed on potato-sucrose agar medium (PSA) in a Petri dishes. After 7 days, the number of fungal colonies grown from the grain was recorded to determine their species affiliations.

The taxonomic status of strains was revealed according to the sum of their macro- and micromorphological characters [18-21]. The species and origin of strains included in the study are shown in table 1. The strains are maintained in the collection of the Laboratory of Mycology and Phytopathology (All-Russian Institute of Plant Protection, St. Petersburg, Russia).

Grain samples were ground separately with a laboratory mill (IKA, Germany), and two samples of flour were selected: a 200 mg sample was used for DNA isolation, and a 1 g sample was used for mycotoxin extraction.

DNA from flour samples was isolated with a reagent kit (TermoFisher Scientific, Lithuania). TaqMan quantitative PCR (qPCR) was performed using specific primers and probes for the detection of the DNA of *Fusarium* fungi producing trichothecene mycotoxins (*Tri-Fusarium*) [22] and the DNA of *Alternaria* fungi [23]. The reactions were carried out in a 20 µl volume containing 10 µl of 2× TaqMan Master Mix (AlkorBio, Russia), each primer at 300 nM, a fluorescent probe at 100 nM (Evrogen, Russia) and 2 µl of the corresponding DNA solution.

Mycotoxins were extracted with 5 mL of an acetonitrile:water mixture (84:16, v/v) for 14-16 h. The amounts of mycotoxins were determined by using certified test-systems for ELISA according to the protocol of the manufacturer (VNIIVSGE, Russia).

Data were analysed by using Microsoft Office Excel 2007 and Statistica 10.0. For the determination of significant differences among samples, a generalized linear mixed model analysis of variance followed by Tukey's test was used. Differences were considered significant at $p < 0.05$.

3 Results and Discussion

The fungal species composition in the mycobiota of the analysed grain samples was abundant and diverse. *Alternaria* and *Fusarium* fungi occurred most often in comparison with other fungi associated with cereal grain harvested in Chechnya.

The other fungi that are usually present in the mycobiota of grain were represented by *Epiccocum*, which was detected in 89.5% of samples, with occurrence ranging from 1-18%; *Nigrospora*, in 63.1% of samples (1-9%); *Aspergillus*, in 52.5% (1-4% of infected grain); and *Arthrinium* in 15,8% (1-3%).

The rate of the infection of grain by *Alternaria* fungi ranged from 55 to 93%. The frequency of occurrence of the species belonging to section *Alternaria* (94% of all *Alternaria* isolates) was much higher than that of the species of section *Infectoriae* (6%).

The infection of grain by fungi of section *Alternaria* was revealed in all grain samples and varied in the range of 52-89%. At the same time, only 78% of the samples

were infected by fungi of section *Infectoriae* (ranging from 1 to 21%). DNA of *Alternaria* fungi was found in all grain samples, accounting for $(4-75) \times 10^{-5}$ % of the total DNA on average, without any differences between grain samples collected in the different biomes (Table 1).

The AOH produced by *Alternaria* fungi was detected in 65% of the samples in amounts of 11-675 ppb.

However, 35% of all analysed grain samples originating from the steppe and dry-steppe zones were free of AOH. Positive correlations between the percentage of grains infected with *Alternaria* section *Alternaria* fungi and the amount of *Alternaria* DNA ($r=0.47$) as well as with the content of AOH ($r=0.46$) were established.

Table 1. The grain infection by *Alternaria* and *Fusarium* fungi and mycotoxins contamination of grain from different climatic zones in the North Caucasus region in 2019

| Climatic zona (number of samples) | Infected grain, % | | Amounts of DNA $\times 10^{-5}$, % of the total DNA | | | Amounts of mycotoxins, ppb | | |
|---|---|-----------------|---|-------------------|--------------|-------------------------------|----------------|-----------------|
| | <i>Alternaria</i> | <i>Fusarium</i> | Tri- <i>Fusarium</i> | <i>Alternaria</i> | DON | T-2 toxin | ZEN | AOH |
| | Occurrence in grain samples, % and (the range of values) | | | | | | | |
| Dry-steppe (n=8) | 100 (54-96) | 12.5 (14) | 37.5 (3-13) | 100 (4-74) | 0 | 25 (15. 650) | 0 | 37.5 (16-42) |
| Steppe (n=5) | 100 (59-96) | 20 (1-2) | 40 (3-6) | 100 (16-46) | 0 | 40 (8. 66) | 20 (14) | 40 (11-102) |
| Forest- steppe (n=8) | 100 (60-94) | 50 (1-8) | 25 (6-29) | 100 (32-75) | 12.5 (40) | 12.5 (7) | 25 (10. 40) | 100 (12-675) |
| Mountain- forest (n=2) | 100 (66-81) | 100 (5-9) | 0 | 100 (33-70) | 0 | 50 (2) | 0 | 100 (30-35) |

The maximum rate of the infection of the grain by *Fusarium* fungi reached 14% in the grain sample of wheat from the dry steppe zone. At least 11 *Fusarium* species were identified in grain: *F. acuminatum*, *F. avenaceum*, *F. equiseti*, *F. graminearum*, *F. langsethiae*, *F. poae*, *F. proliferatum*, *F. semitectum*, *F. sporotrichioides*, *F. tricinctum*, and *F. verticillioides*. DNA of Tri-*Fusarium* fungi was detected in 7 samples, and the amount of fungal DNA ranged from 3.1×10^{-5} to 2.9×10^{-4} % of the total DNA.

Arid conditions in the 2019 growing season resulted in lower infection with *F. graminearum*, which is a typical pathogen of cereals in the North Caucasus region and the main producer of DON and ZEN. This pathogen was detected in 30% of samples, with infection range within 1-6%. DON was found in one grain sample of wheat from the forest-steppe zone at 40 ppb. ZEN was found in three samples of wheat from the steppe and forest-steppe zones at 10-40 ppb. A positive correlation between infection of grain with *F. graminearum* and the amounts of DON and ZEN ($r=0.64$ and $r=0.56$, respectively) was revealed.

Moreover, a positive correlation between the amount of Tri-*Fusarium* DNA and both DON and ZEN amounts ($r=0.88$ and $r=0.79$, respectively) was established. The obtained data are in accordance with the results of our studies carried out previously, in which a strong positive

relationship between *F. graminearum* and its toxic secondary metabolites was demonstrated [24, 25].

The key finding of the study was the identification of *F. langsethiae* in 13% of grain samples of wheat grown in the Chechen Republic, where this species was not previously detected. Recently, *F. langsethiae* was found in wheat grain from the Krasnodar and Stavropol regions, and grain infection by this fungus reached 5% [1].

This species does not cause obvious symptoms of the head, and it is impossible to visually detect the infection of cereals by *F. langsethiae* in the field, in contrast to what is observed for *F. graminearum*, which causes visually apparent symptoms [26]. *F. langsethiae* is a strong producer of the T-2 toxin, as is *F. sporotrichioides* [27-29]. *F. sporotrichioides* was detected in 22% of the samples.

Twenty-six percent of the grain samples were contaminated by the T-2 toxin, in amounts ranging from 7 to 650 ppb. The maximum level of the T-2 toxin was detected in the grain of barley from the dry steppe zone, which exceeded the MPL for this mycotoxin by 6.5 times. In our study, the relationship between the occurrence of *Fusarium* species producing the T-2 toxin and its concentration in grain was not detected, apparently due to the low rate of grain infection by these species (not higher than 3%).

4 Conclusion

Clarification of the species composition of fungi colonizing grain cereals cultivated in a particular region will improve the technology related to the use of contaminated grain for seed, food and fodder purposes. The significant infection of grain with toxin-producing fungi demonstrates the problem of mycotoxins in grain-producing regions of Russia as well as the need to control the quality of raw grain materials used for the production of food and feed.

This study shows that even in a dry growing season, there is a risk of grain contamination with mycotoxins, particularly toxic metabolites of *Alternaria* and *Fusarium* species, which do not require high humidity. At the same time, the presence of pathogenic *F. graminearum* in the grain suggests that in growing seasons with sufficient moisture, the grain contamination by DON and ZEN can be significantly higher than the MPL.

The dynamics and hazards of grain contamination with mycotoxins in each territory can be evaluated only in long-term annual studies, since they are determined not only by the degree of fungal disease spreading but also by the possibility of realizing the toxigenic potential of pathogens, which is influenced by many environmental factors.

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