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## Mycotoxins

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# ADVANCES IN MYCOTOXICOLOGICAL RESEARCH OF FORAGE GRAIN CROPS

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Abstract

Recently, production of forage from the vegetative mass of grain crops has been steadily growing in Russia (Z.L. Fedorova, L.V. Romanenko, 2016; V.V. Popov, 2017; E.A. Volkova et al., 2018). For the successful and safe use of these products, it is extremely important not only to strictly observe the recommended terms, mowing height, drying conditions and technology of silaging grain-stem mass, but also to have the most complete information about the sanitary quality of raw materials. The study of the peculiarities of contamination by toxigenic microscopic fungi and mycotoxins of wild and cultivated cereals has already begun (G.Yu. Laptev et al., 2014; A.A. Burkin, G.P. Kononenko, 2015; G.P. Kononenko et al., 2015; E.A. Yildirim et al., 2019). However, this aspect has not been studied with a focus on forage crops. This work, for the first time, presents data on contamination of vegetative grain crops with toxic metabolites of microscopic fungi and on changes in the content of mycotoxins over phases of plant development and in ears at the beginning of grain maturation. The aim of this work was a mycotoxicological study of common barley (*Hordeum vulgare* L.), soft wheat (*Triticum aestivum* L.), and oats (*Avena sativa* L.) during in the periods optimal for hay harvesting and in unripe ears of wheat and barley. The samples (spring barley *H. vulgare* cv. Vladimir, spring soft wheat *T. aestivum* cv. Ivolga, and oats *A. sativa* cv. Skakun) were collected from April 24 to August 11, 2019 (the fields of the Russian State Agrarian University — Moscow Timiryazev Agricultural Academy and the Williams Federal Scientific Center for Feed Production and Agroecology, Moscow Province). Beginning of tillering—ligule formation was noted as period 1, opening of the flag leaf envelope and appearance of the awns above the ligule—early milk ripeness — as period 2. At the stage of grain maturation, from the aboveground parts cut 3-5 cm from the soil surface the ears were separated. The concentrations of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), roridin A (ROA), aflatoxin $B_1$ ($AB_1$), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR), and ergot alkaloids (EA) were measured by indirect competitive enzyme immunoassay (ELISA) test. The detected load of mycotoxins was generally low. AOL, EMO were present in small and comparable amounts of 15-32 μg/kg and 14-29 μg/kg, as well as CPA and EA with wider ranges of variation, from 34 to 180 μg/kg and from 2 to 115 μg/kg. Fusariotoxins T-2, DON, and ZEN appeared in single samples, and FUM was not detected. ROA was also absent, and PR was extremely rare and detected only in one sample of wheat. In all crops, tens of μg/kg MPA and STE were found, and $AB_1$ amounted to 1-3 μg/kg. Combined contamination of OA and CIT occurred only in barley (more often at tillering and ligule formation), while OA contamination occurred, though rare, in wheat and oats at the levels close to the detection limit. Lower contamination by mycotoxins was characteristic of vegetative oat plants compared to barley and wheat, which is practically important since fodder oat is popular as a green fodder for preservation, both separately and in crop mixtures. Wheat and barley ears at the beginning of grain maturation were noticeably different from the aboveground parts of the plants and showed a uniform tendency to reduce the frequency of mycotoxin detection to single cases or complete absence while maintaining the occurrence of EMO.

Keywords: wheat, barley, oat, plant biomass, mycotoxins, ELISA

Recently, production of forage from the vegetative mass of grain crops

(wheat, barley, oat, and triticale) has been growing in a number of regions of the Russian Federation mainly due to high nutritional value of conservation products thereof and due to the ability to successfully overcome critical situations threatening the reaping of a full grain harvest [1-3]. For the successful and safe use of hay and haylage from the grain crops, it is extremely important not only to strictlyobserve the recommended terms, mowing height, drying conditions and technology of silaging the grain-stem mass, but also to have the most complete information about the sanitary quality of raw materials. Of particular relevance is information about the contamination of crops by toxigenic microscopic fungi and their metabolites resulting in animal mycotoxicoses [4, 5]. For a large community of cereals, the accumulation of such information is just beginning. The abundance of *Fusarium* fungi in pastures of Manitoba province of Canada [6] and Croatia [7] has been studied. The abundance of such fungi and their toxins in 70 species of meadow plants in one of the ecosystems suitable for cattle grazing in Chaco province in northeastern Argentina has been assessed [8, 9]. Systematic affiliation and toxicogenic potential of *Fusarium* fungi from mycobiota of nine species of herbs in five agro-ecological zones of western Iran has been determined [10].

Russian researchers, described the contamination of meadow leguminous grasses with fungi *Fusarium*, *Alternaria*, *Cladosporium* and mycotoxins [11, 12], the features of colonization of wild and cultivated grasses by these fungi [13], the general mycotoxicological situation for the community of meadow plants [14] and industrial mixed sowing of ryegrass, timothy grass, fescue grass, festul lolium and cocksfoot grass [15]. The content of five mycotoxins in samples of ryegrass and timothy grass monocrops has been analyzed [16, 17]. Grain crops harvested without threshing for forage purposes have not been previously studied.

This work, for the first time, presents data on contamination of vegetative grain crops with toxic metabolites of microscopic fungi and on changes in the content of mycotoxins over phases of plant development and in ears at the beginning of grain maturation.

The aim of this work was a mycotoxicological study of vegetative barley, soft wheat, and oats during in the periods optimal for hay harvesting and in unripe ears of wheat and barley.

*Materials and methods.* Specimens of spring barley (*Hordeum vulgare* L.) cv. Vladimir, spring soft wheat (*Triticum aestivum* L.) cv. Ivolga, and oats (*Avena sativa* L.) cv. Skakun were collected from April 24 to August 11, 2019 (the fields of the Russian State Agrarian University — Moscow Timiryazev Agricultural Academy and the Williams Federal Scientific Center for Feed Production and Agroecology, Moscow Province).

Plant phenophases were described according to BBCH classification [18]. Beginning of tillering-ligule (tongue) formation was noted as period I (BBCH 21-39), opening of the flag leaf envelope and appearance of the awns above the ligule-early milk ripeness — as period II (BBCH 49-73). At the stage of grain maturation, from the aboveground parts cut 3-5 cm from the soil surface the ears were separated (waxy ripeness, BBCH 83-87).

The specimens were kept to an air-dry state in a ventilated room and were ground in a laboratory mill M20 (IKA, Germany). For extraction, a mixture of acetonitrile and water in a volume ratio of 84:16 was used at a flow rate of 10 ml per 1 g of the sample. The extracts after 10-fold dilution with buffer solution were used for indirect competitive enzyme-linked immunosorbent assay. The concentrations of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), roridin A (ROA), aflatoxin $B_1$ ($AB_1$), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A

(OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR), and ergot alkaloids (EA) were measured by indirect competitive enzyme immunoassay (ELISA) test (GOST 31653-2012 "Feedstuffs. Method of immunoenzyme mycotoxin determination", M., 2012). The lower limits of quantitative measurements corresponded to 85% level of antibody binding.

The data obtained were processed using the descriptive statistics in Microsoft Excel 2013 program, the proportion of positive samples ($n^+$) from the number of examined samples ($n$), the minimum, maximum content of mycotoxin (μg/kg) and the arithmetic mean value ($M$) for positive specmens was calculated.

*Results.* The studied cereals lacked mycotoxins FUM and ROA. PR was found only in one sample of wheat in quantity of 320 μg/kg. Recently, the similar situation with isolated cases of PR detection and lack of FUM and ROA was described for agrestic annual plants of Cruciferae family [19], as well as for sowing white mustard [20] and sunflower [21]. Unfortunately, information on the contamination of the green mass of corn is still extremely limited. In four studied specimens from the Rostov region, at the stage of three leaves, completion of flowering and physiological ripeness, there were no FUM and ROA (unpublished data of the authors). Nevertheless, it is known that at the beginning of growth, corn and rice are characterized by contamination with fungi *Myrothecium* spp. producing ROA [22].

**1. The occurrence and concentration of mycotoxins in vegetative plants of spring barley (*Hordeum vulgare* L.) cv. Vladimir, spring soft wheat (*Triticum aestivum* L.) cv. Ivolga and oat (*Avena sativa* L.) cv. Skakun** (Moscow Province, 2019)

| Mycotoxin | Barley ($n$ = 32) | | Wheat ($n$ = 11) | | Oat ($n$ = 11) | |
|---|---|---|---|---|---|---|
| | I ($n$ = 14) | II ($n$ = 18) | I ($n$ = 2) | II ($n$ = 9) | I ($n$ = 3) | II ($n$ = 8) |
| T-2 | – | – | – | 2<br>3 | 2<br>2 | – |
| DON | 1<br>95 | – | – | – | – | 1<br>160 |
| ZEN | – | – | 2<br>32 | – | – | – |
| EA | 14<br>7-16-37 | 17<br>2-8-35 | 1<br>3 | 7<br>6-115-425 | 2<br>6 | 4<br>1-2-2 |
| AOL | 14<br>18-30-49 | 18<br>13-29-40 | 2<br>27 | 7<br>16-26-32 | 2<br>32 | 4<br>15-21-25 |
| AB1 | 6<br>2-3-3 | 15<br>2-2-3 | – | 4<br>1-1-2 | – | 2<br>1 |
| STE | 7<br>13-24-25 | 9<br>13-16-22 | – | 4<br>16-20-25 | 1<br>13 | – |
| CPA | 13<br>79-180-320 | 16<br>76-145-265 | 2<br>81 | 9<br>66-140-280 | 2<br>89 | 4<br>27-48-70 |
| EMO | 6<br>11-14-18 | 7<br>11-17-25 | – | 5<br>10-14-19 | 2<br>18 | 6<br>16-29-40 |
| OA | 5<br>6-8-10 | 8<br>4-5-6 | – | 2<br>5 | 2<br>7 | – |
| CIT | 7<br>19-23-33 | 1<br>30 | – | – | – | – |
| MPA | 5<br>19-23-30 | 11<br>13-20-38 | 1<br>21 | 8<br>11-24-40 | – | 3<br>14-18-21 |

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, EA — ergot alkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid. Period I comprises beginning of tillering—ligule (tongue) formation (BBCH 21-39), period II comprises opening of the flag leaf envelope and appearance of the awns above the ligule—early milk ripeness (BBCH 49-73); $n$ is the number of studied specimens. The upper value in lines is the number of positive specimens ($n^+$) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

The most frequently detected mycotoxins in our study were AOL, CPA, and EA, therewith such list was added with MPA and EMO for wheat, EMO for oats, AB1, STE, and MPA. Besides, OA and CIT were regularly found in barley

as opposed to wheat and oats. Fusariotoxins T-2, DON, and ZEN appeared in single specimens (Table 1).

Neither toxin was found in vegetative oat plants, possibly, due to low concentrations close to determination limits. In practical terms, the fact of mild contamination of this culture with mycotoxins is very important, since oats are more often cultivated for green fodder both in pure form and in mixed crops with vetch, tare, and vetchling.

The boundaries of surveillance over the mycotoxicological status of grain fodder crops were selected accounting for the recommended terms of their harvesting for hay (period I, the end of the development of flag leaf at the latest) and for grain silage (period II, completion at the beginning of the milky-wax ripeness of grain).

Based on comparable sets of barley specimens for periods I and II, it is possible to generally assess the direction of changes in mycotoxin contamination (see Table 1). In both periods, the computed average concentration of mycotoxins in positive samples remained comparable. Therefore, concentration of AOL and EMO were equally low (from 14 to 30 μg/kg), contamination by OA was at the baseline, concentration of EA was low, from units to tens μg/kg. Nevertheless, period I differed from period II in a number of CIT, MPA, and $AB_1$ detection instances. At the initial phase, plants were characterized by an increased accumulation of CIT and a decreased accumulation of MPA and $AB_1$. It should be noted that the same tendency were observed for MPA and $AB_1$ in wheat and oats (see Table 1). A variation in the content of mycotoxins by growth periods denotes possible involvement of toxigenic micromycetes in the processes accompanying the change in the ontogenetic states of these plants. However, it is not yet possible to search for any correspondences with the composition of their mycobiota due to the lack of basic information. As is known, mycotoxins are produced by many species of *Alternaria*, *Fusarium*, *Aspergillus*, *Penicillium* genus, as well as other fungi capable of autonomous existence [23-26]. Possibly, some of them are adapted to living inside plants [27-29] and are able to provide the biosynthesis of these metabolites independently or with the participation of the host organism [30].

During period II, concentration of AOL, EMO, and MPA was equally low and stable in barley, wheat, and oats (from 14 to 29 μg/kg), contamination by OA and CIT was baseline or lacked. EA was usually found in small concentrations from units to tens μg/kg, with the exception of a few cases of exceeding the value of 100 μg/kg in wheat (see Table 1). Possibly, it was due to infection of a part of the ears with actively producing epiphytes of the *Claviceps* genus [31]. No differences in occurrence and degree of contamination $AB_1$ (1 and 2 μg/kg, respectively), STE (20 and 24 μg/kg), and CPA (140 and 145 μg/kg) were found in wheat and barley. In barley, $AB_1$ was found even less often, STE was not found, and CPA was by one order less (48 μg/kg). In general, the grain-stem mass in all three crops was characterized by moderate contamination and, according to this indicator, can be recognized as equally promising for laying on grain-silage. Oat cuttings at the late vegetation stages are also suitable for drying, since oat hay retains a high nutritional value at all stages of plant maturity.

In general, in terms of mycotoxin load, the vegetative mass of grain fodder crops differs little from weakly contaminated cruciferous plants and sunflowers [19-21]. The fact that we regularly detected AOL, EA, and CPA in both monocotyledonous and dicotyledonous plants suggests the presence of similarities in the composition of their mycobiota. Indeed, among the endophytes of many plants, fungi with a confirmed potential for AOL and EA biosynthesis have been described [32]; however, no information on the identification of active producers of CPA

[33] in the composition of the internal mycoflora of herbs was found in the available literature.

The differences in the profile of mycotoxins in some plant species, as well as in communities with an annual and interrupted development cycle, indicate the peculiarities in the composition of associated fungi.

In recent years, information on the species diversity of endophytes using molecular methods, in particular, among representatives of *Triticeae* tribe [34], common reed grass *Phragmites australis* (Cav.) Trin. ex Steud. [35], bamboo *Phyllostachys* spp., *Sasa* spp. [36], Siberian cheegrass *Achnatherum sibiricum* (L.) Keng ex Tzvelev [37, 38], and cocksfoot grass *Dactylis glomerata* L. [39] has been actively accumulated. It was established that in cultivated cereals (ryegrass, timothy grass, and wheat) the DNA content of *Alternaria* and *Cladosporium* fungi was 6 times lower and of *Fusarium* 14 times lower than in wild-growing cereals (cocksfoot, wheat grass, timothy grass) [13]. In furtherance, such methods will make it possible to start searching for specific micromycetes responsible for the formation of a natural level of mycotoxin contamination of forage plants, including grain fodder crops.

Ripening ears of wheat and barley significantly differed in the content of mycotoxins from the vegetative biomass of period II (Table 2). T-2, DON, and AOL were more often found in barley, which could be due to fungal infection, since increased contamination with these toxins is observed at intense infection with *Fusarium* and *Alternaria* [40, 41]. Omelchenko et al. [42] noted the relationship between DON accumulation and the maturation of wheat ears affected by the micromycete *Fusarium*.

**2. The occurrence and concentration of mycotoxins in immature ears of spring barley (*Hordeum vulgare* L.) cv. Vladimir and spring soft wheat (*Triticum aestivum* L.) cv. Ivolga** (Moscow Province, 2019)

| Микотоксин | Barley ($n$ = 13) | Wheat ($n$ = 6) |
|---|---|---|
| T-2 | 4 | – |
| | 2-7-10 | |
| DON | 4 | – |
| | 79-150-260 | |
| ZEN | 1 | – |
| | 15 | |
| EA | 2 | 4 |
| | 2 | 3-9-11 |
| AOL | 7 | 2 |
| | 17-74-355 | 16 |
| $AB_1$ | 1 | 2 |
| | 1 | 1 |
| STE | 1 | 2 |
| | 14 | 15 |
| CPA | 1 | 3 |
| | 97 | 32- 44-54 |
| EMO | 4 | 1 |
| | 8- 47-150 | 12 |
| OA | – | – |
| CIT | 1 | – |
| | 24 | |
| MPA | 1/18 | – |

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, EA — ergot alkaloids, AOL — alternariol, $AB_1$ — aflatoxin $B_1$, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, CIT — citrinin, MPA — mycophenolic acid. Period I comprises beginning of tillering—ligule (tongue) formation (BBCH 21-39), period II comprises opening of the flag leaf envelope and appearance of the awns above the ligule—early milk ripeness (BBCH 49-73); $n$ is the number of studied specimens. Upper value in lines is the number of positive specimens ($n^+$) containing mycotoxins in an amount exceeding the lower range limit; concentration of relevant mycotoxin (μg/kg, minimal-mean-maximum) is indicated underneath. A dash means that the mycotoxin was not detected.

In the ears, the occurrence of $AB_1$, STE, CIT, and MPA decreased in several cases or was completely absent (See Table 2). for mature grains of wheat and barley, the situation was similar. Particularly, $AB_1$ was not detected, and the

cases of STE, CIT and MPA detection did not exceed 5% [43]. Contamination of wheat ears by CPA was also significantly lower as compared to biomass (see Table 2), which corresponded to the previously obtained data on its low occurrence (1%) in mature grain [43]. In barley ears, this toxin was found only in one case, and in the grain, it was not found in any of the 92 samples [43]. On the contrary, the frequency of EMO detection remained the same in ripening ears and vegetative mass (30-40%) (see Tables 1, 2), and in threshed grain it reached 72.3% [43]. Since mycotoxin distribution between vegetative parts and ears changes multidirectionally, it can be assumed that the processes of maturation are accompanied by blocking or activation of mycotoxin biosynthesis by associated producers, reformation of the mycobiota composition with their replacement by other fungi or a change in the localization of toxigenic fungi in the plant. Interestingly, in mature corn plants with multiple combined contamination of leaves, stems and flowers, mycotoxin contamination of cobs was not found (unpublished data).

Our characteristics of the main grain fodder crops on the accumulation of fungal metabolites toxic to animals are important for the development of a well-grounded approach to the formation of poly-species herbage, the economic feasibility of which is beyond doubt. Similar projects have yet to be implemented for corn and triticale, as well as for sorghum, which are increasingly being introduced into field fodder production. In the future, we can count on a more detailed description of the mycotoxicological status of crops using a combined high-performance liquid chromatography and mass spectrometry assays [44, 45]. These methods were used to analyze wild-growing grasses in Norway [46] and were successfully applied to assess mycotoxin contamination of the 2017-2018 wheat and barley grain harvest from the Ural region [47].

Thus, mycotoxins slightly contaminated the green mass of wheat, barley and oats, when harvesting early for hay and later for grain silage. It have been established that alternariol, cyclopiazonic acid, emodin and ergoalkaloids regularly occur at a low level; mycophenolic acid and sterigmatocystin contaminate biomass of all cultures; the aflatoxin $B_1$ concentration corresponds to a basal level; fumonisins and roridin A are absent. Wheat and barley ears at the beginning of grain maturation were noticeably different from the aboveground parts of the plants and showed a uniform tendency to reduce the frequency of mycotoxin detection to several cases or complete absence while maintaining the occurrence of emodin. The revealed features of the mycotoxin accumulation during the initial and final plant growth and in ripening ears testify to the active role of toxigenic microscopic fungi in the course of plant development and serve as a convincing argument in favor of the need for complex research projects aimed at interpretation of mechanisms of their interaction with associated fungi.

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