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AFFECTION OF COARSE FODDERS BY TOXIGENIC *Fusarium* FUNGI

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Abstract

Lack of information about the distribution of toxigenic *Fusarium* fungi in hay and cereal straw which is used for bedding and feeding animals inhibits preventive measures against animal toxicoses. Multiple combined fusariotoxin contamination of hay was recently established during advanced mycotoxicological survey of commercially used batches from the livestock farms in European Russia (G.P. Kononenko, A.A. Burkin, 2014). The aim of this work was to study the species complex of fusaric fungi in fodders from Chelyabinsk, Bryansk and Moscow regions of Russia in different years and to evaluate the toxin-producing capacity of their populations. All 60 samples of hay and straw from Chelyabinsk region (1992) were colonized by fusaric fungi of 6 species — *F. sporotrichioides* Sherb, *F. avenaceum* (Corda ex Fr.) Sacc., *F. poae* (Peck) Wr., *F. tricinctum* (Corda) Sacc., *F. culmorum* (W.G. Smith) Sacc. and *F. sambucinum* Fuckel. Unidentified *Fusarium* species were detected in hay of bromegrass, galega, annual (a mixture of wheat, oats and peas) and perennial grasses, and a complex of these fungi was found in rye straw. The majority of samples (90 %) contained *F. sporotrichioides* (a single species, or this species along with other, but *F. sporotrichioides* mostly predominated). Of 19 samples of the hay and straw harvested in the Bryansk region (2011), 16 were infected by *Fusarium* fungi belonging to the same 8 species. *F. sporotrichioides* was found in 11 samples and prevailed, and the other 7 detected species were less frequent — *F. tricinctum* (4 samples), *F. equiseti* (Corda) Sacc. (3 samples), *F. poae* (2 samples), *F. solani* (Mart.) Sacc. (2 samples), *F. culmorum*, *F. graminearum* Schw. and *F. semitectum* Berk. et Rav. (each in one sample). In fodders from Moscow region (2013) *Fusarium* fungi were found in 171 samples of 239 tested (71.5 %) and the affection was often very high. Isolated fungi belonged to the same 8 species as in other regions mentioned. By the frequency of occurrence they were ranged in the following order: *F. sporotrichioides*, *F. tricinctum*, *F. avenaceum*, *F. poae*, *F. culmorum*, *F. graminearum*, *F. semitectum*, *F. solani*, with *F. sporotrichioides* found in 85.7 % of the samples. In addition, we identified three isolates as *F. cerealis* (Cooke) Sacc., *F. sambucinum* and *F. torulosum* (Berk. & M. A. Curtis). Fungal cultures grown on rice grain substrate at 23 °C for 7 days were first used to test the toxin production. *F. sporotrichioides* was shown to possess 100 % potential for T-2 toxin (122-1078 μg/g) and diacetoxyscirpenol (0.7-20 μg/g) production, *F. culmorum*, *F. graminearum*, *F. cerealis*, *F. torulosum* — for deoxynivalenol and zearalenone production, and in two *F. semitectum* isolates the level of zearalenone was high. Extensive distribution of *F. sporotrichioides* producing T-2 toxin and its highly toxic analog diacetoxyscirpenol, necessitates special attention to control the level of these trichothecenes at harvesting and storage of roughage. Specific features of toxin-production of other *Fusarium* species and hay contamination with deoxynivalenol and zearalenone are discussed.

Keywords: hay, straw, *Fusarium* fungi, mycotoxins

High-quality hay and straw are critical for the successful dairy and beef farmers both in temperate climatic zone and tropical areas. There is a significant progress in formation and optimal exploiting grasslands in many countries, but their safe use remains understudied. The reports on roughage contaminated with microscopic fungi capable of toxin production are of great concern. The abundant *Fusarium*, *Aspergillus*, *Mucor*, and *Absidia* microbiota has been found at surveys in Canada and Ireland [1, 2]. When harvesting bermudagrass (*Cynodon* sp.) hay, the major fodder cereal in tropics and subtropics, *Fusarium population* accompanied by *Penicillium*, *Aspergillus*, *Rhizopus* and *Cladosporium* was the most abundant [3].

Until 1980-1990, the feed-caused fusariotoxicoses with sharp positive skin test occurred among calves and sheep in many regions and republics of the USSR, as well as sporadic outbreaks of acute intoxication of ruminants and horses related to "highly toxic fungi" in hay and straw [4-6]. The response measures were unprecedented. Moldy hay and straw with ≥ 10 % contamination, or those infected by *Dendrodochium toxicum* fungus were subject to destroy; the roughages toxic in skin test were prohibited; straw and chaff affected by *Stachybotrys alternans* could only be used after neutralization, and the roughage low-toxic in skin test should not exceed 25 % of animal diets [7].

Fusarium fungi were carefully studied in Ukraine [8, 9], western Belorussia where these species predominated in clover hay [10], in Lithuania and Armenia [11, 12], in the south of Kazakhstan where *Fusarium* fungi were the most abundant on hay and straw [13], in lowland and foothill areas of Dagestan [14]. Nineteen *Fusarium* species and subspecies were found in hay from Northern Ossetia [15], and 13 species were identified on wild fodder cereals from southern Big Caucasus in Azerbaijan [15]. In the Russian Federation the surveys were carried out in Ryazan Province, Volgograd Province [17, 18], Altai [17-19], and then suspended. However, cattle fusariotoxicoses remained a threat. At 1985-1989 Fusarium epiphytotics in crops in Krasnodar Territory, the level of trichothecenes from deoxynivalenol group in the straw exceed that in grain [20]. In the late autumn 1998, acute T-2 toxicosis among calves grazed in the unharvested corn fields in several districts of the Kursk region was due to severe plant infection by fungi (data not published).

Lack of progress in understanding toxigenic fungi prevalence in hay and straw used for bedding or feeding holds back the preventive measures against fusariotoxicoses. Note, the latest data indicate that multiple combined fusariotoxic contamination is characteristic of herbaceous feeds [21-24].

We first in Russia targetedly sought for toxigenic fungi producing trichothecenes and zearalenone in coarse fodders. In the work rice grain substrate was inoculated and cultured for 7 days. This original approach allowed us to identify six *Fusarium* species of 11 fungal isolates as toxigenic ones. Among these, *F. cerealis*, *F. torulosum* and *F. semitectum* are of special interest as toxigenic properties of these species remain rather poorly studied.

Our objectives were to examine fusarium species composition in hay and straw harvested at different times in three Russian regions and to estimate toxigenicity of the revealed fungal populations.

Techniques. Hay and straw batches were sampled from commercial farms in 5 districts of Chelyabinsk Province, 9 districts of Bryansk Province, and 31 districts of Moscow Province in 1992, 2011 and 2013 (Table 1). Each batch was cut into ~2 cm segments, and mixed thoroughly. Part of the batch was placed on Czapek agar supplemented with medical bile (10 %), penicillin (50,000 U/l) and streptomycin (100,000 U/l) in three petri dishes. After incubation at 25 °C for 5 to 7 day the fragments with Fusarium attack were counted, and the portion of affected segments was calculated. For pure isolates, the colonies characteristic of the genus Fusarium, were grown on the same nutrient medium in petri dishes for 5-7 days and then used individually for inoculation of potato glucose agar (PGA) slants. For identification we used taxonomic keys [25] and manuals [26, 27].

To estimate toxin producing activity, approximately equal portions of 10-day PGA fungal culture were individually placed in three 10 ml vials (bottom diameter of 18 mm) with 1 g sterile crushed rice grain pre-wetted with 1 ml H_2O. The vials were closed with cotton-gauze plugs, which were tightly wrapped with a layer of Parafilm M® («Pechiney Plastic Packaging», USA). The vials were kept in the dark for 7 days at 23 °C, then 5 ml mix of acetonitrile:water (v/v 86:14) was added. At the beginning and the end of stationary 14-hour extraction the vials were shaken vigorously.

T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), and zearalenone (ZEN) in the extracts were estimated using certified ELISA test systems [28]. The lower limit of quantification was 0.2 μg/g for T-2, and 0.5 μg/g for the rest fusatiotoxins. We tested *Fusarium* isolates from hay harvested in 2014 in Moscow Province, including *F. graminearum*, *F. culmorum*, *F. semitectum*, *F. solani*, four more isolates with a revised taxonomic identification, randomized sample of *F. sporotrichioides*, *F. poae*, *F. tricinctum*, and *F. avenaceum* (10 isolates of each species), and 12 isolates of *F. sporotrichioides* found in hay with high ZEN contamination.

Data were analyzed in descriptive statistics program Microsoft Excel 2013.

Results. Table 1 shows the characteristics of the samples.

1. Characterization of feeds from different Russian regions tested for *Fusarium* infection

Location, year of harvesting	Feed type, number of batches	Botanical composition (according to batch certificate)
Chellaybinsk Province (Argayashskii, Kaslinskii, Krasnoarmeiskii, Kunashakskii, Sosnovskii regions), 1992	Hay, n = 56	Bromegrass, goat's rue, a mixture of wheat, oats, peas, timothy, fescue, perennial grass mixture, seeded grass mixture, alfalfa
	Straw, n = 4	Cereals (including rye)
Bruansk Province (Bryanskii, Vygonichskii, Zhiryatinskii, Zlynkovskii, Karachaevskii, Klinstovskii, Pochepskii, Trubchevskii regions), 2011	Hay, n = 14	Cereals, meadow grass, ryegrass, herbs
	Straw, n = 5	Not specified
Moscow Province (31 regions, except Krasnogorskii, Naro-Fominskii, Lyuberetskii, Egor'evskii, Stupinskii, Lukhovitskii, Balashikhinskii regions), 2013	Hay, n = 230	Herbs, perennials, meadow mix, timothy, cereals, legumes, bromegrass, alfalfa, fescue, vetch, goat's rue, clover
	Straw, n = 9	Cereals (including wheat and oats), vetch-oats

We found *Fusarium* fungi in all 60 batches of hay and straw from Chelyabinsk Province among which we have identified *F. sporotrichioides* Sherb., *F. avenaceum* (Corda ex Fr.) Sacc., *F. poae* (Peck) Wr., *F. tricinctum* (Corda) Sacc., *F. culmorum* (W.G. Smith) Sacc. and *F. sambucinum* Fuckel. Unidentified Fusaria were revealed in the hay of bromegrass, goat's rue, annual mix (wheat, oats and pea mix), perennial grasses, and in the rye straw. *F. sporotrichioides* was detected in 90 % probes either as a single species, or in combination with with others, but as a rule, the *F. sporotrichioides* prevailed.

In Bryansk Province, among 19 probes of hay and straw tested the 16 ones were attacked by *Fusarium* fungi of 8 species, of these *F. sporotrichioides* was the most frequent (11 probes), while others were more rare, e.g. we identified *F. tricinctum* in 4 probes, *F. equiseti* (Corda) Sacc. in 3 probes, *F. poae* in 2 probes, *F. solani* (Mart.) Sacc. in 2 probes, *F. culmorum*, *F. graminearum* Schw. and *F. semitectum* Berk. et Rav. in one probe each.

In Moscow regions we have identified *Fusarium* fungi in 171 batches form 239 those tested (71.5 %). The degree of sample destruction ranged from 1 to 100 %, making 1-20 % in 47.3 % of the samples and exceeding 50 % in 20 % of the samples.

Isolates from *Arthrosporiella*, *Sporotrichiella*, *Discolor* and *Martiella* sections were allocated to 8 species (Table 2). All these species, also mentioned in two previous reports, were arranged on their predominance in a descending order as *F. sporotrichioides*, *F. tricinctum*, *F. avenaceum*, *F. poae*, *F. culmorum*, *F. graminearum*, *F. semitectum*, *F. solani*.

Similarly to *Fusarium* fungi in Chelyabinsk and Bryansk provinces, the prevalence rate for *F. sporotrichioides* was high and reached 85.4 % vs. 8-15 % for *F. tricinctum*, *F. avenaceum* and *F. poae*, 2.3 % for *F. culmorum* and *F. graminearum*, 1.2 % for *F. semitectum*, and 0.6 % for *F. solani*. For some species no compliance of their occurrence with the botanical composition of hay and the place of harvesting was traced, however, in three cases of four *F. graminearum* detection this species was found in timothy hay from western areas of the region (see Table 2).

2. Prevalence of *Fusarium* fungi species in hay and straw (Moscow Province, 2013)

Section	*Fusarium* species	Prevalence, % (n = 171)	Number of isolates
Arthrosporiella	*F. sporotrichioides* Sherb.	85,4	265
	F. avenaceum (Corda ex Fr.) Sacc.	11,1	20
	F. semitectum Berk. et Rav.	1,2	2
Sporotrichiella	*F. tricinctum* (Corda) Sacc.	15,2	28
	F. poae (Peck) Wr.	8,2	15
Discolor	*F. culmorum* (W.G. Smith) Sacc.	2,3	4
	F. graminearum Schw.	2,3	4
Martiella	*F. solani* (Mart.) Sacc.	0,6	1
-	*Fusarium* spp.	4,7	9

N o t e. n — the number of isolates tested.

Four of the found *Fusarium* spp. isolates (see Table 2) were attributed according to modern taxonomic systems and approaches. As a result, the isolate No 252/3, earlier morphologically described as *F. culmorum* according to Booth system, was identified as *F. cerealis* (Cooke) Sacc., and the isolate No. 392/1, previously attributed to *F. graminearum*, was identified as *F. torulosum* (Berk. & M.A. Curtis). Of two fungal species which could not be initially identified the isolate No. 79/4a belonged to the *F. incarnatum-equiseti* species complex (FIESC), and the isolate No. 79/4b was attributed to *F. sambucinum* Fuckel. The remaining five *Fusarium* spp. isolates (see Table 2) were contaminated by *Penicillium* fungi, which complicated the research and eventually led to loss of the fungal cultures.

Toxigenicity was assessed in *F. sporotrichioides*, *F. tricinctum*, *F. avenaceum* and *F. poae* (10 isolates of each species), and in all other species cultures. For rapid evaluation, a procedure which provided stable active growth of compact culture was developed: fungal cultures grew for 7 days at 23 °C on crushed rice grain as a substrate (1 g), sterilized after moistening (1 ml of water), in 10 cm^3-vials with cotton plugs, tightly wrapped with a layer of laboratory film to prevent drying out.

3. Toxigenicity (μg/g of substrate) **of *Fusarium sporotrichioides* after 7-day growth on crushed rice grain at 23 °C** (n = 3, $X \pm s$)

Isolate No.	T-2	DAS	ZEN
9/1	327±48	1.7±0.4	–
50/3	348±154	1.4±0.3	0.5±0.1
54/1	290±70	1.4±0.2	1.2±0.4
236/1	408±76	1.8±0.4	–
276/2	396±8	3.6±0.2	–
324/2	1078±353	20.0±3.0	–
349/4	446±110	2.5±0.6	–
501/1	387±146	2.1±0.7	–
614/3	122±66	0.7±0.2	0.7±0.2
647/3	362±141	2,3±1,0	–

N o t e. T-2 — T-2 toxin, DAS — diacetoxyscirpenol, ZEN — zearalenone; n — number of replications in the experimrnt, X — arithmetical mean, s — sample mean error. Dashes mean the mycotoxin is not detected.

All 10 *F. sporotrichioides* isolates produced T-2 (122-1078 μg/g) and DAS (0.7-20 μg/g), and only a few ones could produce ZEN (3 of 10 isolates, 0.5-1.2 μg/g) (Table 3). Apparently, the cases of acute dermatitis in cows, when the *F. sporotrichioides*-infected oat straw has been used, were caused by the T-2 toxin [29]. We have revealed the extensive hay lesion by *F. sporotrichioides*, the active producer of T-2 and its highly toxic analogue, in different territories and in different years. Hence, a special attention should be paid to control of the T-2 content during harvesting and feed storing.

4. Toxigenicity (μg/g of substrate) **of *Fusarium culmorum*, *F. cerealis*, *F. graminearum*, and *F. torulosum* after 7-day growth on crushed rice grain at 23 °C** (n = 3, $X \pm s$)

Isolate No.	DON	ZEN
	F. culmorum	
28/3	5±1	57±55
258/4	55±5	256±95
266/1	10±3	88±26
505/1	9±1	12±7
	F. cerealis	
252/3	16±4	14±7
	F. graminearum	
9/3	37±21	18±8
109/2	3±1	3±1
389/1	23±14	80±79
398/1	49±29	2±1
	F. torulosum	
392/1	62±21	101±65

Note. DON — deoxynivalenol, ZEN — zearalenone; n — number of replications in the experimrnt, X — arithmetical mean, s — sample mean error.

F. poae, *F. avenaceum*, *F. tricinctum* did not produce T-2 and ZEN. Importantly, the toxigenicity of *F. sporotrichioides* and *F. poae* in a rapid test on a grain substrate coincided with the results under prolonged fungal culture [30-32].

In most *F. culmorum* and *F. graminearum* cultures isolated from hay, as well as in sporadic isolates of *F. cerealis* and *F. torulosum*, DON and ZEN amounts were more than 10 μg/g (Table 4).

However, it is unlikely that the total contribution of the trichothecenes of the DON and ZEN groups to the contamination of coarse fodder can be significant due to the weak fungal affection. Nevertheless, the researchers in Germany [33] and Canada (2) reported about a combined hay contamination. An extensive T-2, HT-2, DON and ZEN contamination of cereals and straw occurred at Swedish farms in 2011-2012 [34].

Low DON production (0.6±0.1 μg/g) was characteristic of isolate No. 79/4a from *F. incarnatum-equiseti* complex, whereas ZEN was not found. No toxins were not detected in *F. solani* (No. 537/4) and *F. sambucinum* (No. 79/4б), however, *F. semitectum* isolates No. 252/4 and No. 373/4 of different samples actively produced ZEN (77±13 and 110±5 μg/g, respectively).

Grain substrate for fungal culture, first applied in this work, allowed a comparative assessment of the toxicity of representative samples, which is especially important when working with natural populations. Our results showed (see Tables 3 and 4) that the relative sample mean error at 3-fold replications was 34 %, and for T-2 and DAS it was less (28 %) than for ZEN (44 %). Thus, it is necessary to improve this methodology.

Finding the agents caused ZEN contamination in hay remains very relevant. In Ireland, this toxin was found in 21 % of hay samples [2]. Previously we found high ZEN accumulation in the hay, harvested in the European Russia, including the Moscow region [21-24]. Six ZEN-contaminated probes contained 12 *F. sporotrichioides* strains, and two of these probes contained *F. culmorum* (No. 266/1), *F. tricinctum* (No. 266/3) and *F. semitectum* (No. 373/4). Only 3 of 12 *F. sporotrichioides* isolates excreted low amounts of ZEN, *F. tricinctum* (No. 266/3) did not produce ZEN, the toxin-producing activity of *F. culmorum* (No. 266/1) was similar to that of other members of the genus (see Table 4), and only *F. semitectum* (No. 373/4) was highly active. Interestingly, in the hay from which the No. 373/4 was isolated the ZEN amount reached 3160 μg/kg, and, when compared to the collection *Fusarium* strains on potato-sucrose agar, this species was classified as a possible ZEN producer [24].

Obviously, the mycological analyses of plants and feeds can not exhaustively reflect complex and dynamic processes in mycobiota, so the probability of identifying the causal relationships leading to an increased risk of fusariotoxicosis, is not so great. Nevertheless, the determination of the species composition and the toxigenic potential of the entire complex of *Fusarium* fungi affecting coarse fodder remains important, since high-toxic isolates are found among the new species [35]. Mycotoxicological evaluation of roughage should be continued not only for fusarium fungi. The ability to produce ZEN and its derivatives is known for the phytopathogenic fungus *Drechslera portulacae* [36] and fungi of the genus *Stachybotrys* [37]. Using a moist chamber technique, we did not find fungi of highly toxic cellulose destructors — *Stachybotrys alternans* and *Dendrodochium toxicum* (= *Myrothecium roridum*) in hay and straw samples from the Chelyabinsk region, nevertheless, fodder contamination by toxic metabolites is of special relevance. In recent years, new information about the prevalence of such fungi inhabiting soils and plant residues has appeared. Recently, mycobiota of maize phylloplans in the Primorsky Territory have been replenished with the *Myrothecium verrucaria* fungus, which can infect plants in the early ontogenesis [38].

Thus, among the fusarium fungi affecting hay and straw, six species are able to provide a combined contamination with mycotoxins — T-2 and DAS (*Fusarium sporotrichioides*), DON and ZEN (*F. graminearum*, *F. culmorum*, *F. cerealis*, *F. torulosum*), and ZEN (*F. semitectum*). The extensive *F. sporotrichioides* infection, found in different territories and in different years, specially indicates the need to control the amount of T-2 and its highly toxic analogue, DAS, during harvesting and storage. It is rational to elucidate the causes of ZEN contamination in hay, taking into account the effects of biogenic and anthropogenic factors, as well as the possible contribution of fungi belonging to other taxonomic groups. The rapid fungal culture on grain substrate, first applied in this work and allowing a comparative assessment of the toxicity of fungi in a representative sample sets, seems to be promising for the investigation of natural populations.

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