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SPECIES COMPOSITION AND TOXICOLOGICAL CHARACTERISTICS OF FUNGI OF THE GENUS *Aspergillus* ISOLATED FROM COARSE FODDERS

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Abstract

The problem of ensuring the safety of coarse fodders, which annually replenishes the Russian feed production on a large scale, raises concern about the multiple combined contamination with mycotoxins and the extensive spread of toxigenic fungi. Recently, it has been established that *F. sporotrichioides* plays a dominant role among fusarium fungi in these fodders, producing metabolites, the T-2 toxin and diacetoxyscirpenol, which can cause acute poisoning in animals. The purpose of this work, which became the next stage in the study of the main toxin-producing micromycetes of coarse fodders, was the determination of the species composition, occurrence and toxin production in fungi of the genus *Aspergillus* under experimental conditions favourable for the fullest realization of their potential. The objects of mycological analysis were 258 average samples from the production batches of hay and straw harvested in the livestock farms of Bryansk (2011) and Moscow (2013) regions. Isolates with established species affiliation were cultivated on Czapek-Dox agar (CDA), wort agar (WA) and moistened rice grain (RG) for 7 days at 23 °C. The content of sterigmatocystin (STE), emodin (EMO), aflatoxin B_1 (AB_1), ochratoxin A (OA), mycophenolic acid (MPA), cyclopiazonic acid (CPA), ergot alkaloids (EA), deoxynivalenol (DON) and fumonisins (FUM) in extracts of mycelial spore biomass were determined by enzyme immunoassay with certified test systems. To assess the toxin production, 32 isolates of 12 species of *Aspergillus* fungi of hay and straw were used, as well as 27 isolates of *A. flavus* Link, *A. pseudoglaucus* Blochwitz, *A. repens* de Bary isolated earlier from grain fodders. Fungi of the genus *Aspergillus* were found in samples with a frequency of 62.0 % and an infection rate of 1.7-100 %. The obtained isolates belonged to 15 species included in 10 taxonomic groups with the largest species diversity in the *A. glaucus* group (4 species). The most common species were *A. flavus* and *A. niger* van Tieghem (more than 50.0 % of the contaminated samples), followed by *A. versicolor* (Vuill.) Tiraboschi, *A. pseudoglaucus, A. amstelodami* (Mangin) Thom & Church, *A. ochraceus* Wilhelm and *A. wentii* Vehmer (10.6-18.8 %), *A. nidulans* Eidam (6.3 %), the remaining 7 species — *A. candidus* Link, *A. tamarii* Kita, *A. sydowii* (Bain. & Sart.) Thom & Church, *A. fumigatus* Fresenius, *A. repens*, *A. terreus* Thom, *A. chevalieri* (Mangin) Thom & Church were less common (< 5 %). The intensity of formation of the CPA (*A. flavus*) and MPA (*A. pseudoglaucus, A. repens*) was quite comparable in the CDA and RG. Compared to WA, a greater accumulation of the majority of mycotoxins occurred in the RG, i.e. STE (*A. versicolor, A. nidulans*), CPA (*A. flavus*, in all 5 *A. tamarii* isolates CPA could be detected only on this substrate) and EMO (*A. sydowii*). For the biosynthesis of MPA in *A. pseudoglaucus* and *A. repens*, WA was preferred. Testing of fungi on three nutrient media allowes us to establish that a complex of *Aspergillus* fungi which includes 7 species can be associated with the contamination of coarse fodders with STE, CPA and MPA; the source of EMO contamination among the fungi of the genus *Aspergillus* was not found. Only two of the three isolates of *A. sydowii* produced it in small amounts of 120±20 and 245±40 ng/g. The remaining mycotoxins analyzed in the isolates were not detected. The possibility of participation of fungi of other systematic groups in the contamination of fodder with STE, CPA, MPA and EMO is discussed, whereas clusters encoding the biosynthesis of mycotoxins have been found in micromycetes from genetically distinct groups in recent years.

Keywords: hay, straw, fungi of the genus *Aspergillus*, mycotoxins

Micromycetes of the genus *Aspergillus* have the negative impact on ani-

mals not only by mycoses caused by pathogenic species [1, 2], but also via intoxication by fungal metabolic products with a wide spectrum of damage, including neurotoxicity with tremorgenic effect, hepato- and nephrotoxicity [3, 4]. Despite numerous reports on significant fungal contamination of fodder, the risks associated with aspergillotoxicosis are still unclear. In the world literature, information about toxin production of some fungal species of this genus can be found, but as rule, they were obtained for a small number of isolates, under different conditions and often for one or a few closely related toxins [5, 6]. All these make it difficult or impossible to even approximately analyze situations which might arise in animal feeding and care.

Annually, coarse fodders noticeably replenish Russian forage reserve, and it causes concern of experts because of multiple combined mycotoxins contamination [7, 8] and wide spread of *Aspergillus, Penicillinu, Fusarium* and many other fungi, including toxigenic species. Predominance of *F. sporotrichioideds*, which metabolic products are T-2 toxin and diacetoxyscirpenol capable to cause acute toxicosis, has recently been found among fusarium in coarse fodders [9].

We were the first to targetedly seek for toxigenic fungi of the genus *Aspergillus* among species involved in damaging hay and straw in different Russian regions. A new approach to testing isolates on alternative growth media allowed us to confirm possibility of mycotoxins biosynthesis in 7 *Aspergillus* species of 15 identified in the mycobiota. The rear species such as *A. tamarii*, *A. repens* and *A. wentii* is a main focus of interest because information about their toxic potential is quite lacked.

The purpose of this work was to assess species composition of genus *Aspergillus* in coarse fodders and toxigenicity of these isolates in laboratory tests when ensured full realization of their potential.

Techniques. The mycological analysis was performed for 258 bulk samples of hay and straw commercial batches from livestock farms. These were 14 hay samples (cereals, meadow grasses, ryegrass, herbs) and 5 straw samples (the composition was not specified) from Bryansk region (8 areas, 2011), and 230 samples of hay (herbs, cereal, perennial grasses, composed, prairie grasses, timothy grass, bromegrass, lucerne, fescue grass, cereal-legume, vetch, goat's rue, clover) and 9 straw samples (vetch-oats mixture, cereal, wheat, oat) from Moscow region (31 areas, 2013). Inoculum was prepared as described previously [9]. Each batch was cut into 2 cm fragments, and mixed vigorously. The fragments were placed in three Petri dishes, 20 pieces in each per bach, with approximately the same distances between them. Czapek agar supplemented with medical bile (10 %) and antibiotics (penicillin 50,000 IU and streptomycin 100,000 IU per 1 liter of medium). The dishes were placed in a thermostat at 25 25 °C. After 5-7 days, the percentage of the total number of fragments with *Aspergillus* attack was calculated. To isolate pure cultures, the colonies having appearance and features of genus *Aspergillus* were seeded on the same agar medium in Petri dishes, and in 5-7 days, after confirming the purity, reseeded on Czapek Dox Agar (CDA). Species identification was carried out according to identification keys of fungi [10].

Estimation of toxigenicity included preparation of inoculum, substrate, seeding, culture, extraction mycotoxins and their analysis. The 10-day fungi cultures were used to seed on CDA. An approximately equal amount of inoculum, taken from the agar surface using mycological crochet, were placed in three 10 ml vials, a bottom diameter of about 18 mm, with 1 g sterile crushed rice grain prewetted with 1 ml H_2O, and also in three vials with 1.5 ml of CDA or wort agar (WA) (Liofilchem, Italy). The vials were closed with cotton-gauze plugs, which were tightly wrapped with a layer of Parafilm M® (PM-996, Pechiney Plastic Packaging, USA). The vials were kept in the dark for 7 days at 23 °C. Then, acetonitrile:water mixture (v/v 86:16) was added in each vial, and at the begin-

ning and the end of stationary 14-hour extraction the vials were shaken vigorously. The content of sterigmatocystin (STE), emodin (EMO), aflatoxin B_1 (AB_1), ochratoxin A (OA), mycophenolic acid (MPA), cyclopiazonic acid (CPA), ergot alkaloids (EA), deoxynivalenol (DON) and fumonisins (FUM) in extracts were estimated using certified ELISA test systems [11], the lower limit of detection corresponded to 85 % antibody binding. For estimating toxigenicity, 32 isolates of 12 *Aspergillus* species from hay and straw were used, as well as 27 isolates of *A. flavus*, *A. pseudoglaucus*, *A. repens* from the collection of All-Russian Research Institute of Veterinary Sanitary, Hygiene and Ecology which have been isolated earlier from grain forages.

Obtained data were analyzed by descriptive statistics method in Microsoft Excel 2013 software. The tables show the arithmetic mean values (X) and the errors of sample mean (s).

1. Species composition and prevalence of *Aspergillus* fungi in coarse forages (hay and straw) harvested in Bryansk Province (8 regions, 2011) **and Moscow Province** (31 regions, 2013) (n = 160)

Group	Species	Frequency of occurance, %
A. flavus	*A. flavus* Link	56.3
	A. tamarii Kita	3.1
A. niger	*A. niger* van Tieghem	54.4
A. versicolor	*A. versicolor* (Vuill.) Tiraboschi	18.8
	A. sydowii (Bain. & Sart.) Thom & Church	2.5
A. glaucus	*A. pseudoglaucus* Blochwitz	15.6
	A. amstelodami (Mangin) Thom & Church	10.6
	A. repens de Bary	1.9
	A. chevalieri (Mangin) Thom & Church	0.6
A. ochraceus	*A. ochraceus* Wilhelm	15.0
A. wentii	*A. wentii* Vehmer	14.4
A. nidulans	*A. nidulans* Eidam	6.3
A. candidus	*A. candidus* Link	4.4
A. fumigatus	*A. fumigatus* Fresenius	2.5
A. terreus	*A. terreus* Thom	0.7
Не определена	*Aspergillus* spp.	3.8

N o t e. n — number of samples affected by aspergillus.

Results. *Aspergillus* fungi were found in 62.0 % of 258 tested samples with an infection rate from 1.7 to 100 %. Pure cultures of the fungal isolates of this genus were assigned to 15 species of 10 taxonomic groups (Table 1). The highest species diversity was in the *A. glaucus* group with 4 species (*A. pseudoglaucus*, *A. repens*, *A. amstelodami*, *A. chevalieri*), the others comprised 1-2 species. We failed to identify isolates of 13 samples at a species level because they were lost in the early stages of isolation, but according to the pre-estimates the 7 of them belonged to the *A. glaucus* group.

By frequency of occurrence, *A. flavus* and *A. niger* were among the leaders (more than 50.0 % of the affected samples), they were followed by *A. versicolor*, *A. pseudoglaucus*, *A. amstelodami*, *A. ochraceus* and *A. wentii* (0.6-18.8 % of samples), *A. nidulans* (6.3 %), and the rest 7 species, *A. candidus*, *A. tamarii*, *A. sydowii*, *A. fumigatus*, *A. repens*, *A. terreus* and *A. chevalieri* were noticeably more rare (< 5 %). Generally, these results coincided with those obtained earlier in other territories — in the Ryazan Meshcher [12], Tatarstan [13, 14], Dagestan [15], North Ossetia [16] and the Amur Region [17], as well as in the ex-USSR republics — Ukraine [18], Belarus [19], Lithuania [20], Armenia [21, 22], Kazakhstan [23] and Azerbaijan [24, 25]. All surveys reported that *Aspergillus* fungi dominate in the mycobiota of coarse fodders and are represented by many species with a predominance of *A. flavus* and *A. niger*. The similar pattern, although with some differences, was also found for the accompanying species *A. fumigatus*, *A. nidulans*, *A. ochraceus*, *A. versicolor*, *A. candidus*, *A. wentii,* and *A. glaucus*. Among rare species, the researchers found *A. clavatus* [16, 18, 21, 25], *A. flavipes* [22], *A. oryzae* and *A. ustus* [16].

Apparently, the complex of *Aspergillus* species and the observed ratio between them were the result of long-term competitive interrelationships between fungi and formed during the grass stand drying. It is difficult to assume that such a

variety of vegetative plants are subjected to fungal attack so uniformly. It is clear that different species could actively develop on living plants, and those that eventually dominated, during the growing season, could be of secondary importance. In our view, this fact and the suggestion above deserve special attention, since they point to the need for accounting the biosynthetic potential of not only common, but also rarely identified species for the correct prediction of the feed contamination risks.

Toxigenic estimate methodology for this fungi group, according to the available information, was not the subject of special studies. However, in other works, the Czapek-Doks medium was used for testing and producing mycotoxins [26], the assessment of *A. ochraceus* isolates was carried out on rice grains [27, 28], and some fungal species were differentiated by ability of the isolates to accumulate EMO [29], CPA [30, 31] and MPA [32] on WA. In tests on WA, CDA and moistened rice grain (RG), three toxigenic species from the collection (Table 2) under equal conditions (7 days, 23 °C) showed similar production of CPA (*A. flavus*) and mycophenolic acid (MPA) (*A. pseudoglaucus* and *A. repens*) on rice and CDA in all cases, whereas on WA, the CPA production by *A. flavus* isolates was much weaker.

2. Toxigenicity of *Aspergillus* fungi isolated from grain forage on wort agar (WA), **Czapek-Doxa agar** (CDA) **and moistened rice grain** (RG) (23 °C, 7 days)

Species of fungus (number of isolates)	Mycotoxin	Amount of mycotoxin, X±s ng/g substrate		
		WA	CDA	RG
A. flavus (6)	CPA	125±28	1410±280	900±165
		200±80	1780±40	1710±200
		330±55	1190±110	1055±270
		–	93±15	205±83
		200±45	945±245	780±195
		260±78	810±155	1115±180
A. pseudoglaucus (2)	MPA	nd	970±69	1425±95
		nd	775±46	1040±56
A. repens (3)	MPA	nd	975±155	1145±150
		nd	1060±145	1560±190
		nd	2560±335	3005±920

N o t e. CPA — cyclopiazonic acid, MPA — mycophenolic acid; X is the arithmetic mean, s is the sample mean error. A dash indicates that mycotoxin is not found, nd — no detection permormed.

3. Toxigenicity of *Aspergillus* fungi isolated from coarse fodders on wort agar (WA)**, and moistened rice grain** (RG) (23 °C, 7 days)

Species of fungus (number of isolates)	Mycotoxin (n^+)	Amount of mycotoxin, X±s ng/g substrate	
		WA	RG
A. versicolor (3)	STE (3)	1060±150	197860±30560
		160±32	41620±6520
		4050±810	223960±43310
A. pseudoglaucus (4)	MPA (4)	30320±5620	21730±4340
		1750±130	685±44
		1840±45	630±75
		2200±310	940±22
A. wentii (3)	MPA (1)	123±22	–
A. nidulans (3)	STE (3)	–	21460±4200
		–	9480±1830
		–	13570±2330
A. tamarii (5)	CPA (5)	–	3410±680
		–	1920±255
		–	1370±105
		–	2780±550
		–	1760±270
A. sydowii (3)	EMO (2)	–	120±18
		–	245±45
A. repens (2)	MPA (2)	1020±42	480±27
		810±98	595±80

N o t e. STE — sterigmatocystin, CPA — cyclopiazonic acid, MPA — mycophenolic acid, EMO — emodin; n+ — number of isolates producing mycotoxin; X is the arithmetic mean, s is the sample mean error. Dashes mean that mycotoxin is not found.

Given differences in the metabolic response revealed on WA, we continued hay and straw testing on WA and RG (Table 3), of which RG was previously used for growth *Fusarium* from the same objects [9]. The analyzed mycotoxins were AB_1, STE, OA and EA which production by *Aspergillus* fungi is rather well studied [35], MPA, CPA and EMO [29-32] which are less studied, and also FUM and DON which are found only in several strains of *Aspergillus* [33, 34]. For analyses, we selected three to four isolates of five widespread species and *A. nidulans*, and all the isolates obtained (from 1 to 5) were tested for more infrequent species. Pathogenic *A. candidus* and *A. niger* isolated from feed were unable to produce the mycotoxins in question so they were excluded from further study. The choice of two growth medium for testing toxigenicity, as it turned out, was a success. The RG medium allowed for higher accumulation of STE (*A. versicolor*, *A. nidulans*), CPA (*A. tamarii*, only this substrate made it possible to detect CPA in all 5 isolates) and EMO (*A. sydowii*). On the contrary, for the MPA biosynthesis in *A. pseudoglaucus* and *A. repens*, the WA medium was preferable. As per the estimates of *Aspergillus* fungi, the relative error of the mean sample in 3-fold repetition did not exceed 20 % for WA and RG and was quite acceptable.

The tests showed that a complex of *Aspergillus* fungi which comprises 7 species isolated from coarse fodders is capable for biosynthesis of STE, CPA and MPA. *A. flavus* and *A. tamarii*, the two members of the *A. flavus* group, can produce CPA, *A. versicolor* and *A. nidulans* may synthetize STE, and *A. pseudoglaucus*, *A. repens* and *A. wentii* are MPA-producing isolates. Mycotoxicological analysis of hay and straw from the same regions detected an extensive STE, MPA, CPA and EA contamination with incidents of significant accumulated amounts [7, 8]. Potential EMO contaminants of fodder were not found among *Aspergillus* fungi. Only two of three *A. sydowii* isolates produced EMO in small amounts of 120±20 and 245±40 ng/g. *A. fumigatus*, which metabolome contains EA and EMO [36], was represented by a single isolate producing EA in the amount of 220±32 ng/g and not capable of EMO synthesis. OA, FUM and DON were not found in mycelial and spore biomass. CTE was accompanied by trace concentrations of AB_1, 5±3 and 14±1 ng/g, in two *A. versicolor* isolates when grown on RG. *A. amstelodami* and *A. ochraceus*, the members of common forage species, and also *A. terreus* and *A. chevalieri* which are rare in occurrence, did not form any of the studied mycotoxins in amounts exceeding tens of nanograms per 1 g of substrate.

While recognizing the toxigenic potential of *Aspergillus* species for fodder contamination by mycotoxins, it should be noted that, according to the accepted criteria [37], these species basically belong to the weak producers, as the accumulation of toxins, even under the most favorable conditions, did not reach 10,000 ng/g substrate in all *A. pseudoglaucus* isolates and *A. nidulans* and was of next lower order in *A. flavus* and *A. tamarii*. Only one of the frequently occurred species, *A. versicolor,* showed itself as a highly active producer of STE when cultured on RG. Perhaps, fungi of another systematic groups, in particular from the genus *Penicillium*, may participate in forage contamination with the same toxins. Previous studies have indicated that not only representatives of *Aspergillus*, but also fungi of other genera are capable of forming many toxins [38, 39]. Therefore, in the light of modern concepts, definition “aspergillotoxins” becomes more and more conventional. The development of genome analysis shows that clusters encoding the biosynthesis of a particular mycotoxin actually occur in fungi of genetically distant groups [33].

The present paper provides data on the ability of *Aspergillus* fungi, the members of coarse fodder mycobiota, to toxin formation, although not always significant. Nevertheless, it is quite possible that the realization of potential toxygenicity could be enhanced, not only in vivo, during grass growing, but also after

mowing, drying and storing. The study of the causes of abrupt shifts in the metabolic profile in fungi under the influence of habitat conditions noted for certain sanitary species, in particular for *Fusarium graminearum* Schw., remains in focus of mycological research.

Thus, for 7 species of the genus *Aspergillus*, the possibility of participation in extensive contamination of feeds by cyclopiazonic acid, sterigmatocystin and mycophenolic acid with cases of accumulation of significant amounts of mycotoxins has been confirmed. However, the sources of contamination with other toxins, in particular emodin, ochratoxin A, ergoalkaloids, could well be among the representatives of the other identified species, and among those of unidentified taxonomic attribution. Because of the small number of available isolates, we were unlikely to fully appreciate the toxin-forming ability of *A. tamarii*, *A. sydowii*, *A. fumigatus*, as well as *A. terreus* and *A. chevalieri*. It is possible that for *A. amstelodami* and *A. ochraceus*, the selected substrates were not entirely suitable for inducing toxin formation. It would therefore be advisable to extend the search for carriers of toxicity among these species of *Aspergillus* and other micromycetes from the mycobiota of coarse forages and also to continue testing on different media for an exhaustive estimation of the fungal potential of toxigenicity.

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