SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2015, V. 50, № 2, pp. 255-262 ISSN 2313-4836 (Online)

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UDC 636.085.19:636.086.1/.3:632.4(470)

doi: 10.15389/agrobiology.2015.2.255rus
doi: 10.15389/agrobiology.2015.2.255eng

ABOUT ZEARALENONE LEVELS IN GRASS FODDERS AND TOXINE PRODUCING ACTIVITY OF *Fusarium* FUNGI

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Supported in part by Russian Science Foundation (project № 14-16-00114)
Received September 3, 2014

Abstract

Zearalenone, the *Fusarium* fungi metabolite with an estrogenic effect, can lead to significant economic losses in livestock due to fodder contamination. Pasture herbage and fodders from dried pasture plants can really cause intoxication. However, the reasons and mechanism of abnormally high accumulation of zearalenone in these fodders are not still found out. In this article we summarized the data on investigation of frequency and the level of zearalenone contamination in wild herbage and in hay. Also the ability to produce this mycotoxin was studied in vitro in 13 *Fusarium* strains from laboratory collection. Hay samples from different farms of 30 regions in Moscow Province were prepared from 120 hay batches in 2013. A total of 211 samples of wild herbage including (*Calamagrostis*, dogstail grass, *Anthoxanthum*, reed canary grass, hedgehog, fire, bluegrass, fescue, bentgrass, bluegrass, timothy, foxtail) were collected in Kashirskii, Noginskii and Ruzskii regions of Moscow Province in 2014 from June to September. A total of 427 samples of pasture grasses were also repeatedly collected in North Karelia, Leningradskaya, Tverskaya and Astrakhanskaya provinces from 1998 to 2014 at different frequency for different plant species. In these samples, along with commonly grown grasses such as *Bromus*, bluegrass, fescue, bluegrass, timothy, some local plants were found, particularly reed, reed canary grass, hedgehog, bent grass, reeds, Leymus, dogtail grass, oats, ryegrass, clover, rank, peas, vetch, rye, pampasskaya grass, foxtail, alfalfa. An uneven zearalenone contamination of pasture grasses with rare overcontamination cases was shown to occur to the end of vegetation. In hay an increased frequency and more high level of contamination were found. The mycotoxin was detected in 54 samples of 120 those tested, and more than a half of them contained zearalenone at the level below 100 μg/kg, while the rest samples contained toxin at 1000 μg/kg, and in 13 % of the samples tested the contamination reached 1000 to 10000 μg/kg. After 7 day cultivation of *F. graminearum*, *F. equiseti* and *F. semitectum* on semi-synthetic agar medium more that 80 % of strains produced zearalenone, and the rate of strains with the high production reaching more than 10000 ng/ml was 5, 15 and 44 %, respectively. *F. cerealis*, *F. culmorum*, *F. sporotrichioides*, *F. poae* seemed to be less active producers, and among *F. anguioides* and *F. langsethiae*, as well as *F. heterosporum*, *F. chlamydosporum* and *F. kyushuense* the zearalenone producers were not found. In a strain of *F. flocciferum* which was tested a high biosynthetic ability and accumulation of zearalenoine at 15 500 ng/ml were detected. An involvement of *Fusarium* fungi having more pronounced metabolic response to environment changes in contamination of grass fodder with zearalenone is discussed. Also the possibility of forecasting hay contamination basing on pasture survey is under consideration.

Keywords: grasses, fodders, hay, zearalenone, *Fusarium* fungi.

As an «estrogenic food factor», Zearalenone (ZEN) produced by *Fusarium* fungi poses a serious threat to animal health. Situations with mass damage of reproductive functions were described in 1970-1980's for ruminants, horses and pigs [1, 2], and similar cases continue to be reported [3]. Frequent occurrence of ZEN in dried grasses was found in Serbia [4] and later in Ireland [5]. In addition, cases of very intensive accumulation (up to several thousands of micrograms per 1 kg of dry material) were noted [1, 4]. We observed a similar situation for hay from farms in European Russia where

ZEN amount was lower than 100 μg/kg in the majority of samples, but sometimes it exceeded 1,000 μg/kg [6]. Although a real risk of livestock intoxications exists when such fodders are used, insufficient attention has been paid to identification of reasons for so high contamination.

It cannot be excluded that the effect of abnormal accumulation could be caused by the process of herbage drying on ground. In practice, its conditions are dissimilar and vary in duration from several days to weeks, depending on weather and economic/organizational reasons. Therefore, in order to understand this phenomenon, we first shall compare feedstock contamination before harvesting and after drying.

Our work was aimed at investigating zearalenone occurrence and content in grass stands of grain and legume crops in the summer-autumn period and in hay of various botanical composition, as well as at making a comparative assessment of toxigenic capacity of *Fusarium* fungi which infect agricultural plants.

Technique. Mean hay samples were taken from 120 hay batches harvested at different farms in 30 districts of the Moscow Province in 2013. A total of 211 samples of wild plants, including bluejoint, dog's-tail, vernal grass, baldingera grass, cocksfoot, bromegrass, bluegrass, fescue, bentgrass, couch, timothy, bristlegrass, were collected in the Kashirsky, Noginsky and Ruzsky Districts of the Moscow Province from June to September of 2014. A total of 427 samples of meadow grasses were also collected in North Karelia, Leningrad, Tver' and Astrakhan provinces from 1998 to 2014 with different frequency by years and plant species. Along with ubiquitous grasses (bromegrass, bluegrass, fescue, couch, timothy), these samples included the locally distributed plants suach as bluejoint (everywhere, except in North Karelia), baldingera grass (North Karelia, Leningrad Province), cocksfoot (Leningrad and Tver' Province), bentgrass (North Karelia, Tver' Province), and reed (Tver' and Astrakhan provinces), as well as the plants found in individual territories, such as lyme grass (North Karelia), dog's-tail, oat, ryegrass, clover, vetchling, pea, vetch (Tver Province), rye, pampas grass, bristlegrass, medick (Astrakhan Province).

Freshly harvested grass samples were dried at 50 °C, scissored and then ground using a laboratory mill. The prepared material was extracted with a mixture of acetonitrile and water in the volume ratio 86:14; extraction agent consumption was 10 ml per 1 g of sample weight.

The study involved 13 species of *Fusarium* fungi from the collection of the Laboratory of Mycology and Phytopathology of the All-Russian Research Institute of Plant Protection (St. Petersburg). The inoculum was represented by 10-12-day monosporous fungal culture grown on potato sucrose agar. A 1 ml portion of potato sucrose agar was added into sterile flat-bottomed glass vials closed with cotton-gauze plugs, and then the fungus was inoculated. To prevent growth medium drying, vial plugs were wrapped with Parafilm (USA). After 7 days (upon completion of cultivation), 1 ml of acetonitrile-water mixture in the volume ratio 86:14 was added into each vial, and the vial contents were thoroughly shaken at the beginning and at the end of stationary 14-16-hour extraction. In the comparative assessment of toxigenic capacity of *Fusarium* species in and differentiation of cultures based on toxin production rate, we used the terminology proposed in specialist literature [7, 8].

ZEN content was determined using a commercial test system for indirect competitive enzyme-linked immunoassay, Zearalenon-IFA (Russia), that provided the lower limit of detection at 20 μg/kg in plant objects and 4 ng/ml in mycelium and spore biomass of fungi (9).

Results. Throughout the observation period from June to September, ZEN was found in vegetative meadow grasses at a rate of 18 % (38 of 211 sam-

ples), and its quantity was generally below 100 μg/kg. However, in 18 % of positive samples, its content was substantially higher with the maximum achieved value of 5,760 μg/kg (Table 1). Interestingly, gradual accumulation of this mycotoxin was observed by the months of collection with its occurrence rate varying from 10.6 to 27.3 %. Thus, ZEN content was below 100 μg/kg in June. In July, we noted some cases when values exceeded 100 μg/kg, and in August and September we found samples containing more than 1,000 μg/kg of this fusariotoxin. In other words, the nonuniform pattern of contamination with ZEN with rare cases of ultrahigh accumulation was established in meadow grasses by the end of the vegetation period. Significant ZEN content was revealed in couch, timothy and bluegrass, which says for a mycogenic nature of this phenomenon as opposed to a phytogenic one.

1. Zearalenone occurrence rate and content in meadow grasses and harvested hay (Moscow Province, 2013-2014)

Object, collection time, number of samples	*n*+	*n*+, Zearalenone content		
		20-100 μg/kg	> 100 μg/kg	> 1,000 μg/kg
Meadow grasses (*n* = 211):	38	31 (25-99)	3 (117-146)	4 (1,060-5,750)
June, *n* = 92	16	16 (27-56)	–	–
July, *n* = 55	15	12 (52-99)	3 (117, 126, 146)	–
August, *n* = 47	5	2 (25, 26)	–	3 (1,060, 2,000, 5,760)
September, *n* = 17	2	1 79	–	1 1,520
Hay, *n* = 120	54	30 (20-89)	17 (100-870)	7 (1,320-10,000)

N o t e : *n* is the number of analyzed samples, *n*+ is the number of toxin-containing samples. Dash means that no positive samples were found. Zearalenone content (minimum-maximum) is indicated in brackets.

Based on the results of analysis of samples from 120 production batches, a hay contamination pattern turned out to be similar to that previously described for a composite sample from various territories [6]. In general, contamination with the mycotoxin was moderate, however, individual cases of high toxin accumulation were noted. In total, ZEN was found in 45 % of samples, and more than a half of them contained less than 100 μg/kg. Mycotoxin quantity in the other samples was by an order of magnitude higher, and it exceeded 1,000 μg/kg in 13 %. In one sample of perennial grass hay, this parameter achieved 10,000 μg/kg. A case of such ultrahigh grain contamination with ZEN was previously described by Finnish scientists [1]. In Slovenia, this toxin was observed in dried corn plant stand at within 200-700 μg/kg, being occasionally over 10,000 μg/kg [4].

The results obtained for material from the same territory in different seasons indicated that, in general, the contamination pattern for vegetative grasses and those harvested for fodder had similar features, i.e. low ZEN content was predominant with medium occurrence indices; at the same time, significant mycotoxin quantity was noted in some samples.

The condition of grass stands in the second half of the vegetation period in a specific territory may serve as a guide in assessing the risk of dry grass fodder contamination with this toxin. The data obtained for other areas are of interest in this regard. Thus, the analysis of wild grasses from North Karelia and Leningrad Province (*n* = 177) did not reveal any case of ZEN detection. In the Tver' Province, in July of 2011 and July and September of 2014, contamination was low reaching 45-105 μg/kg only in 12 of 100 samples. Conversely, in the Astrakhan Province (*n* = 150), high contamination of reed (767; 3,020 and 10,000 μg/kg) was noted as early as in the first yields in September of 1999-2000. Few positive samples were revealed in 2005 and 2009. Unfortunately, we were un-

able to carry out similar surveys in the Bryansk Province, where a high level of hay contamination (up to 2,000 μg/kg) was found for the first time [6]. It should be noted that representative samples of the plants forming the basis of natural local grass stands are necessary for prediction of a specific situation in each territory.

In spite of obvious similarity between vegetative plants and those harvested for fodder, we observed an increase in ZEN detection rate, as well as in the share of samples with high degree of contamination and maximum accumulation. This is probably associated with the fact that metabolism of the fungi capable of mycotoxin biosynthesis becomes active in the process of wilting and drying. In particular, L.E. Taffarel et al. [10] have demonstrated that ZEN content is higher in the grass dried in the sun with rapid change of day and night temperatures, rather than in the shade [10].

Plants can be infected by many *Fusarium* species. Their toxigenicity is covered in many studies [11, 12], however, the potential of ZEN formation is not fully understood yet, and any firm conclusions are premature. Evaluative experiments were mainly carried out in case of fungus growth on sterilized grain substrates at 15-27 °C within several weeks. It has been established that this toxin was produced with various rates by representatives of 8 species: *F. graminearum* Schwabe, *F. culmorum* (W.G. Sm.) Sacc., *F. sporotrichioides* Sherb., *F. poae* (Peck) Wollenw., *F. arthrosporioides* Sherb., *F. avenaceum* (Fr.) Sacc., *F. langsethiae* Torp et Nirenberg, *F. tricinctum* (Corda) Sacc., which are most common in grain in Finland [13, 14]. However, producers were not found among *F. culmorum*, *F. equiseti* (Corda) Sacc. and *F. avenaceum* strains isolated from winter wheat seedlings in the Moscow Province [15]. As for the species infecting grain in the same region, small amounts of ZEN were revealed only in 3 of 11 isolates of *F. culmorum,* and no producers were identified among *F. avenaceum*, *F. sporotrichioides* and *F. poae*. At that, *F. sporotrichioides*, *F. poae*, *F. equiseti*, *F. acuminatum* Ellis et Everh. within mycobiota of grain from other regions were characterized by low accumulation of this metabolite [17, 18]. Among 76 *F. graminearum* isolates which were obtained from fusarium grain grown in the Krasnodar Territory, more than a half of them produced ZEN [19].

In recent years, a new alternative approach becomes increasingly frequent in comparative assessment of fungal toxin production. It consists in short-term cultivation of strains on liquid or agarized media [8]. One of such alternatives (cultivation on potato sucrose agar within 7 days at 23-24 °C) was used in this work. A total of 13 *Fusarium* species were selected for the study; for 9 of them, sample size was from 14 to 74 strains. According to the results, most of *F. graminearum* representatives (61 of 74) produced 23–41,200 ng of ZEN per 1 ml of medium (Table 2). Their distribution was uneven within this range, and the share of the isolates containing over 10,000 ng/g of the mycotoxin was equal to 5 %. The toxin production potential of *F. equiseti* was 100 % with the rate of 50–39,810 ng/ml and increase in high-activity strain number up to 15 %. The species *F. semitectum* Berk. & Ravenel [syn. *F. incarnatum* (Desm.) Sacc.] with about the same potential as for *F. graminearum* (90.0 % versus 82.4 %) was characterized by the substantially higher share of the highly active producers (44 %) which were able to give the mycotoxin in the quantity of over 100,000 ng/ml.

In case of *F. cerealis* (Cooke) Sacc. and *F. culmorum*, ZEN detection frequency was quite high, and ZEN content generally ranged from 100 to 1,000 ng/ml. In the *F. sporotrichioides* and *F. poae* samples comparable by size, only few strains produced this toxin, and, as a rule, its concentrations were low (10-40 ng/ml). No producers were identified among the *F. an-*

guioides Sherb. (n = 20) and *F. langsethiae* (n = 17).

2. The Zearalenone quantity produced by collection stains of *Fusarium* in case of 7-day cultivation on potato sucrose agar at 23-24 °C (laboratory experiment)

Fusarium species	$n+/n$	$n+$, Zearalenone quantity			
		10 ng/ml	100 ng/ml	1,000 ng/ml	10,000 ng/ml
F. graminearum	61/74	12 (2–70)	32 (105–813)	14 (1,100–7,080)	3 (11,480, 12,880, 41,200)
F. equiseti	14/14	1 50	4 (170–830)	7 (1,050–3,470)	2 (12,590, 39,810)
F. semitectum	27/30	-	11 (130–880)	4 (1,260–5,125)	12 (16,220–181,970)
F. cerealis	18/26	4 (30–80)	13 (100–795)	1 (1,260)	–
F. culmorum	15/16	5 (40–80)	10 (157–616)	–	–
F. sporotrichioides	3/43	2 (11, 19)	1 (190)	–	–
F. poae	3/46	3 (10, 12, 40)	–	–	–

Note: n is the number of analyzed samples of fungal mycelium and spore biomass, $n+$ is the number of Zearalenone-containing samples. Dash means that no positive samples were found. Zearalenone content (minimum-maximum) is indicated in parentheses.

No ZEN was found in few cultures of *F. heterosporum* Nees et T. Nees (n = 6), *F. chlamydosporum* Wollenw. & Reinking (n = 2) and *F. kyushuense* O'Donnell & T. Aoki (n = 2) which were available for the study. High biosynthesis activity with regard to this metabolite was detected in the only one studied strain of *F. flocciferum* Corda (15,500 ng/ml).

Based on the obtained results, potential sources of ZEN accumulation in grasses may include *F. graminearum*, *F. equiseti* and *F. semitectum.* Interestingly, *Fusarium* sp. isolates were obtained from the reed heavily contaminated with ZEN in the Astrakhan Province, and they supposedly belong to *F. graminearum* and actively produce this metabolite both on grain substrate and agarized media. The isolate of the same species was also obtained as a result of a mycological analysis of a hay sample containing 2,000 μg/kg of the toxin from the Bryansk Provincce. Earlier, it was demonstrated that incubation temperature decrease to 22 °C leads to significant ZEN accumulation capacity in case of *F. graminearum* [20]. Probably, it is especially susceptible to change of metabolic pathways under the influence of development conditions. This problem may be clarified by the recent investigation of gene mechanisms behind ZEN biosynthesis regulation in *F. graminearum* [21, 22].

The clarification of species composition and biosynthetic capabilities of *Fusarium* fungi represented in the grass mycobiota is still an objective of future studies. In this regard, it is very important to identify constituent organisms, taking into account the latest advances in chemotaxonomic and molecular genetic differentiation of fungi [23, 24], as well as to study the factors influencing the activation of the corresponding genome regions.

Thus, for vegetative meadow plants and hay, we have found similarities and differences in respect to occurrence rate and content of Zearalenone, a fusariotoxin with estrogenic effect. Also, we have denoted the basic methodological approaches required for determination of causes for intensive mycotoxin accumulation in grass fodders. The solution of this problem will make it possible to eliminate or substantially reduce the threat of mycogenic intoxications leading to impairment of reproductive function in animals.

Acknowledgements

The authors are grateful to the management of the Central Veterinary Office of the Moscow Region and the staff of local animal disease control stations for organization

of hay sampling for the study.

REFERENCES

1. Kallela K., Ettala E. The estrogenic fusarium toxin (zearalenone) in hay as a cause of early abortions in the cow. *Nordisk Veterinaermedicin*, 1984, 36(9/10): 305-309.
2. Di Menna M.E., Lauren D.R., Poole P.R., Mortimer P.H., Hills R.A., Agnew M.P. Zearalenone in New Zealand pasture herbage and the mycotoxins-producing potential of *Fusarium* spp. from pasture. *New Zealand J. Agric. Res.*, 1987, 30(4): 499-504 (doi: 10.1080/00288233.1987.10417963).
3. Wallgren P., Sjölund M., Hulten C., Nordkvist E., Häggblom P. Increased occurrence of *Fusarium* mycotoxins in this year's harvest. *Svensk Veterinärtidning*, 2011, 63(15): 35-37.
4. Kordic B., Panin M., Kandic S., Lončarevic A., Muntaňola-Cvetkovic M. Results of a three year microbiological and mycotoxicological examination of feeds in SR of Serbia. *Proc. II Simpozijum o mikotoksinima*. Sarajevo, 1986: 17-28.
5. Buckley T., Creighton A., Fogarti U. Analysis of Canadian and Irish forage, oats and commercially available equine concentrate feed for pathogenic fungi and mycotoxins. *Ir. Vet. J.*, 2007, 60: 231-236 (doi: 10.1186/2046-0481-60-4-231).
6. Kononenko G.P., Burkin A.A. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2014, 4: 120-126 (doi: 10.15389/agrobiology.2014.4.120rus, 10.15389/agrobiology.2014.4.120eng).
7. Varga J., Rigo K., Lamper C., Teren J., Szabo G. Kinetics of ochratoxin A production in different *Aspergillus* species. *Acta Biologica Hungarica*, 2002, 53(3): 381-388.
8. Kononenko G.P., Burkin A.A. *Immunologiya, allergologiya, infektologiya*, 2010, 1: 195-196.
9. Burkin A.A., Kononenko G.P., Soboleva N.A. *Prikladnaya biokhimiya i mikrobiologiya*, 2002, 38(3): 305-311.
10. Taffarel L.E., Mesquita E.E., Castagnara D.D., Costa P.B., Neres M.A., Horn M.B., Oliveira P.S.R., Meinerz C.C. Dehydration curve, fungi and mycotoxins in Tifton 85 hay dehydrated in the field and in shed. *Revista Brasileira de Zootecnia*, 2013, 42(6): 395-403 (doi: 10.1590/S1516-35982013000600003).
11. Marasas W.F.O. Toxigenic fusaria. In: *Mycotoxins and animal foods*. J.E. Smith, R.S. Henderson (eds.). Boca Raton, USA, 1991: 119-139.
12. Leslie *J.F.,* Summerell *B.A. The Fusarium laboratory manual*. Blackwell, 2006.
13. Jestoi M., Paavanen-Huhtala S., Uhlig S., Rizzo A., Yli-Mattila T. Mycotoxins and cytotoxicity of Finnish *Fusarium* strains grown on rice cultures. *Proc. 2nd Int. Symp. on Fusarium head blight incorporating the 8th European Fusarium Seminar*. S.M. Canty, T. Boring, J. Wardell, R.W. Ward (eds.). Orlando, FL, USA, 2004: 405-409.
14. Jestoi M., Paavanen-Huhtala S., Parikka P., Yli-Mattila T. In vitro and in vivo mycotoxin production of *Fusarium* species isolated from Finnish grains. *Arc. Phytopath. Plant Protect.*, 2008, 41(8): 545-558 (doi: 10.1080/03235400600881547).
15. Kononenko G.P., Malinovskaya L.S., Piryazeva E.A., Soboleva N.A. *Mikologiya i fitopatologiya*, 1998, 32(4): 37-41.
16. Kononenko G.P., Malinovskaya L.S., Soboleva N.A., Piryazeva E.A., Zotova E.V., Shalyganova O.N. *Mikologiya i fitopatologiya*, 1999, 33(2): 118-124.
17. Kononenko G.P. *Sistema mikotoksikologicheskogo kontrolya ob"ektov veterinarno-sanitarnogo i ekologicheskogo nadzora. Avtoreferat doktorskoi dissertatsii* [The system for mycological and toxicological control of the objects of veterinary, sanitary and environmental surveillance. DSc Thesis]. Moscow, 2005.
18. Tokarev S.V., Kononenko G.P., Soboleva N.A., Burkin A.A. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2009, 1: 89-92.
19. Leonov A.N., Malinovskaya L.S., Soboleva N.A., Kononenko G.P. *Doklady VASKHNIL*, 1990, 11: 21-26.
20. Leonov A.N., Kononenko G.P., Soboleva N.A., Malinovskaya L.S. *Mikologiya i fitopatologiya*, 1994, 28(1): 60-63.
21. Kim J.E., Han K.H., Jin J., Kim H., Kim J.C., Yan S.H., Lee Y.W. Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zeae*. *Mol. Microbiol.*, 2005, 58: 1102-1113.
22. Lisøe E., Klemsdal S.S., Bone K.R., Flandsen R.J.N., Johansen T., Thrane U., Giese H. The PKS4 gene of *Fusarium graminearum* is essential for zearalenone production. *Appl. Environ. Microbiol.*, 2006, 72(6): 3924-3932 (doi: 10.1128/AEM.00963-05).
23. Kosiak E.B., Holst-Jensen A., Rundberget T., Gonzalez J.M.T., Torp M. Morphological, chemical and molecular differentiation of *Fusarium equiseti* isolated from Norwegian cereals. *Int. J. Food Microbiol.*, 2005, 99: 195-206.
24. Gagkaeva T.Yu. V sbornike: *Mikologiya segodnya. Tom 2* [In: Mycology today. V. 2]. Moscow, 2011: 14-29.