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A TYPICAL MYCOTOXIN PROFILE FOR ORIGINAL (REPRODUCTIVE) OILSEED RAPE SEEDS

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Abstract

For rapeseed, the world's third largest source of vegetable oil, there are special requirements for post-harvest processing, which are rapid drying to the required moisture content due to the threat of mass mold during storage (J.T. Mills, 1987; J.T. Mills, R.N. Sinha, 1980). The authors of publications explained all known cases of mycotoxin detection in seeds of this crop by either infection of plants in the field or by the impact of unfavorable factors during harvesting (I. Brazauskiene et al., 2006; A. Mankeviciene et al., 2011, L. Wu et al., 2017). In this study, for the first time, we have confirmed that the presence of toxic metabolites of fungi of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Myrothecium* and a number of others is not typical for seeds of this crop. The purpose of this work was mycotoxicological examination of oilseed rape *Brassica napus* L. ssp. oleifera (Metzg.) Sinsk seeds under proper phytosanitary and technological conditions during cultivation, harvesting and storage. Original (reproduction) seeds were harvested in 2009-2021 from experimental plots (the Laboratory of fodder crops and field feed production systems, the Williams Federal Research Center, Moscow Province). After grinding in a laboratory mill, 158 samples were analyzed by a unified methodology using certified commercial and research enzyme immunoassay test systems (GOST 31653–2012). For extraction, a mixture of acetonitrile and water was used (84:16 v/v), 5 ml per 1 g sample. Mycotoxins (T-2 toxin, deoxynivalenol, zearalenone, fumonisins of group B, ergot alkaloids, alternariol, roridin A, aflatoxin B_1, sterigmatocystin, cyclopiazonic acid, emodin, ochratoxin A, citrinin, mycophenolic acid, PR-toxin) were determined in extracts after 10-fold dilution with phosphate-salt buffer solution (pH 7.4) with Tween 20. The analyzed mycotoxins were not found in the seeds of the 2009-2020 harvests collected under normal weather conditions and without violations of the drying and storage regimes. For only one sample of spring rapeseed (Bison variety, 2019), weak contamination with mycophenolic acid was detected and the producer *Aspergillus pseudoglaucus* Blochwitz was identified in the mycobiota. In the seeds of the 2021 harvested under elevated temperature and humidity, contamination with ergot alkaloids (from 2 to 12 μg/kg = ppb) was found in 67.5 % of spring and 25.6 % of winter crops. In addition, 28.6 % of spring rape samples contained alternariol, mainly in low concentrations (from 8 to 32 ppb) and rarely with a more pronounced accumulation (from 46 to 775 ppb). In one spring rapeseed sample which was stored in an under-dried state for the longest time, the greatest contamination with alternariol occurred and intensive infection with the fungus *Alternaria tenuissima* (Nees et T. Nees:Fries) Wiltshire were determined with an experimentally confirmed ability to toxin formation.

Keywords: oilseed rape, seeds, mycotoxins, enzyme immunoassay

For seeds of oilseed rape, the world's third-largest source of vegetable oil, accounting for 14.1% of global production in 2020-2021 [1], there is a special post-harvest requirement for drying as quickly as possible to a standard moisture

content of 5-8%. This is associated with a real threat of mass molding of seeds, which is especially relevant in zones with a warm and humid harvesting period, as well as in storage areas where deviations from optimal parameters are observed [2, 3]. Studies performed in Romania [4, 5], Lithuania [6, 7], Poland [8, 9], Serbia [10, 11], India [12, 13], Ethiopia [14] showed that at harvesting and during storage of the rape seeds there is an extensive community of associated fungi, including both pathogens of these plants and saprotrophic fungi. Information on the contamination of rape seeds with mycotoxins is scarce. Researchers explain their appearance either by infection of plants in the field, or by the impact of unfavorable factors during harvesting [15, 16].

In this study, for the first time, confirmation is presented that the presence of toxic metabolites of fungi of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Myrothecium* and a number of others is not typical for rapeseed seeds.

The purpose of the work is mycotoxicological examination of oilseed rape seeds obtained in compliance with the appropriate phytosanitary and technological conditions during cultivation, harvesting and storage.

Materials and methods. The objects of the study were 158 samples of original (reproductive) seeds of winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk from the experimental plots of the Williams Federal Scientific Center for Forage Production and Agroecology (VIK) in 2009-2021.

Comparison of data on air temperature and humidity during seed harvesting was carried out based on data of the Lugovaya meteorological station (Moscow Province).

Mycotoxicological analysis of seeds of the 2021 harvest (120 samples) was carried out 2 months after harvest. Seeds of the 2020 harvest and samples of previous collections taken from the seed warehouse were analyzed in March 2021, that is, the shelf life was about 9 months (harvest 2020) and from 2 to 10 years.

The content of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), group B fumonisins (FUM), ergoalkaloids (EA), 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) were determined according to the unified method (GOST 31653-2012). Feeds. Method for enzyme immunoassay for the determination of mycotoxins. Moscow, 2012) using a panel of 15 certified commercial and research enzyme immunoassay test systems (STO 00494143.01-2015 Test systems for indirect competitive enzyme immunoassay. General specifications, VNIIVSGE). The lower limits of measurements corresponded to 85% antibody binding and amounted to 1 (AB_1, EA), 2 (T-2, OA, STE), 5 (ROA), 10 (AOL, MPA, ZEN, EMO, CIT, CPA), 40 (DON, FUM), 100 μg/kg (PR).

For the extraction of ground seed samples, a mixture of acetonitrile and water was used in a ratio of 84:16, 5 ml per 1 g sample. Indirect competitive enzyme immunoassay was performed after 10-fold dilution of the extracts with phosphate-buffered saline (pH 7.4) with Tween 20.

Results. The data on the rape seed samples used in the work are shown in Table 1. When using a unified methodology and a panel of 15 enzyme immunoassay test systems, no mycotoxins were detected in 38 rapeseed seed samples from the 2009-2020 crops.

Their complete absence indicated that this state remained stable and did not depend on the seed storage time. During harvesting in these years, weather conditions corresponded to long-term climatic parameters, and no deviations from the standards for drying seeds were recorded. It should be noted that earlier, in five samples of oilseed rape seeds harvested in 2015-2018 from eastern Poland, none of the 13 analytes were fund, including fusariotoxins diacetoxyscirpenol, T-

2 toxin, HT-2, nivalenol, deoxynivalenol, 3-acetyl- deoxynivalenol, fusarenone X, zearalenone, as well as aflatoxins B1, B2, G1, G2 and ochratoxin A [17].

1. Characterization of spring and winter oilseed rape *Brassica napus* L. ssp. oleifera (Metzg.) Sinsk seeds used in mycotoxicological examination (Moscow Province, 2009-2021)

Crop	Year	Varieties, hybrids, breeding samples
Winter rapeseed	2012-2018	Varieties: Horizon (2018), Severyanin (2012, 2013, 2017, 2018), Capital (2014, 2017, 2018)
	2019	Varieties: Garant, Severyanin
	2020	Variety: Severyanin
	2021	Varieties: Garant (two sowing dates), Horizont (two sowing dates), Dobrodey, Kazimir, Laureate, Loris, Nord (two sowing dates), Olivine, Progress, Sarmat, Severyanin (two sowing dates), Severyanin (another reproduction), Seligor, Capital (two sowing terms), Elvis, Asparagus, Brauen Schnitthole, Dunne, Grunder Sch., Hangry Gap, Imperial, Jot Neuf, Jrig Scedrap, Lider, Symmons, Taisetze note, Victor, Zenit, Zorni Samples: No. 4, No. 2 NPTs, No. 4 NPTs, No. 6 NPTs, VN-360-14L, VN-364-14R, VN-469-15L, VN 726-17R, VN 912-15
Spring rapeseed	2009-2018	Varieties: Bizon (2016), Vikros (2011, 2017), Grant (2009), Lugovskoy (2017), Novosel (2015), Podmoskovny (2012, 2015, 2018)
	2019	Varieties: Bison, Veles, Grant, Novosel, Podmoskovny
	2020	Varieties: Vikros, Novosel, Podmoskovny, Svetozar
	2021	Varieties: Bison, Veles, Vikros, Grant, Lugovskoy, Novik, Novosel, Podmoskovny Hybrids: Astra 4, Astra 5, Astra 6, VIK 1, VIK 2, VIK 3, VIK 4, VIK 5, Vikros, Drago, Karalino, Lagonda, Casket, Lumen, Miracle, Macro, Salsa M 45, Smilla, Chevy KL Samples: Vikros M 48.2, Vikros MD 38, Vikros MD 69.1, Vikros MD 70, Vikros ML 35, Vikros ML 38, Vikros ML 49, Vikros ML 69, Vikros ML 102, Grant MD 4.1, Novik MA 81, 25-3, 379-13, 557-15, 580-15, 902, 951-3, 948-3, 948-4, 948-6, 949-2; 951-1, 951-18, 7, 10, 15, 17, 19, 23, 26, 27, 38, 39, 40, 1/20, F 1/21, 2-7/15 M 74, 8/20 , 18/20, 29/20, 32/20, 33/20, 75/20, 88/20, 359/20, 369/20, 372/20, 388/20

The only exception for the period from 2009 to 2019 was a sample of spring oilseed rape (variety Bizon, 2019) which contained MPA at a low concentration close to the limit of detection (20 μg/kg). One of the strains of *Aspergillus pseudoglaucus* Blochwitz (No. 448/2) detected by mycological analysis of the sample according to common procedure including isolation and identification of pure cultures, produced MPA (2000 ng/g) and an anthraquinone toxin EMO (70 ng/g) during express testing (7 days, 25 °C, wort agar). The appearance of MPA in this sample was probably the result of a short-term violation of its storage conditions, and a longer exposure to negative factors could lead to combined contamination. The production of two toxins with a quantitative predominance of MPA is typical for strains of this fungus found in grain products (unpublished data of the authors).

The seeds of the 2021 harvest also lacked T-2, DON, ZEN, FUM, OA, CIT, AB_1, STE, CPA, PR, EMO, and ROA. However, EA and AOL were found in some samples (Table 2). EA, the biosynthesis of which is known for fungi of many taxa, including the genera *Penicillim* and *Aspergillus* [18], were contained at a basal amounts of 2 to 12 μg/kg, and the frequency of their detection in spring varieties was higher than in winter varieties (67.5 % vs. 25.6%). AOL, a toxic metabolite of small-spore *Alternaria* species, was detected in 28.6% of spring oilseed rape seed samples. The content of this toxin mostly was low, from 8 to 32 μg/kg, only in 6 samples, it varied from 46 to 775 μg/g. Most of the positive samples contained only EA (32 samples) or were co-contaminated (20 samples), two had only AOL.

The 2021 growing season was generally warmer than usual. In 34 decades of the year out of 37, the average daily air temperature was above the norm. The average daily temperature exceeded the long-term average in summer by 4.6 °C, in autumn by 3.1 °C, the growing season lasted until the end of the first decade

of November. Precipitation was 1.36 times more than the annual averages, and the excess was unevenly distributed from April to September.

2. Mycotoxin occurrence ($n+$) and accumulation in original (reproduction) seeds of spring and winter rapeseed *Brassica napus* L. ssp. oleifera (Metzg.) Sinsk (Moscow Province, 2021)

Crop	n	n^+ (mycotoxin concentration min-max, μg/kg)	
		ergoalkaloids	alternariol
Winter rapeseed	43	11 (2-12)	—
Spring rapeseed	77	52 (2-5)	22 (8-775)

N o t e. n is the number of samples examined, $n+$ is the number of positive samples containing mycotoxins in an amount exceeding the lower limit of measurements. A dash means that the mycotoxin was not detected.

The harvesting of winter seeds at the end of July 2021 took place under more favorable conditions than spring ones: the increase in precipitation in July was the least and amounted to 13.6% of the monthly norm. Spring seeds were collected at the end of the growing season as they matured, when the humidity exceeded the norm by 70.9% (in August) and 52.8% (in September). The samples contained many weed seeds, the moisture content of which turned out to be especially high, and it was not always possible to rapidly dry seeds to the standard moisture content. The combination of these circumstances, apparently, caused the active growth of toxigenic micromycetes and the accumulation of mycotoxins in seeds, which is more pronounced in spring crops. One of the samples of spring rapeseed, designated as F 1/21 (see Table 1), which was not dried to the normative humidity, along with EA (3 μg/kg) contained AOL in the largest amount, 775 μg/kg. Mycological analysis revealed intense (more than 50%) infection with *Alternaria tenuissima* (Nees et T. Nees: Fries) Wiltshire. Three isolates (Nos. 456/1, 456/2, and 456/4) during cultivation (7 days, 25 °C, malt agar) confirmed the ability to produce AOL in amounts of 980, 1520, and 2500 ng/g of medium.

Previously, for fungi found in oilseed rape seeds, the toxic effects of metabolites of *Alternaria* spp. were revealed [7] and aflatoxigenicity was found in three isolates of *Aspergillus flavus* [19]. The species *Aspergillus flavipes* found in the composition of endophytes of oilseed rape stems is also potentially toxigenic [20]. The search for micromycetes involved in the contamination of the seeds of this crop with toxic metabolites should be continued within the framework of more detailed mycological examinations.

The generalization of the obtained results showed that, subject to the phytosanitary and technological rules, regardless of the type of cultivation of oilseed rape, there were no mycotoxins in its seeds. This new fact allows us to assume their extremely low content in vegetative plants. Earlier, it was shown that in sunflower, the contamination of achenes is residual compared to the green mass [21], and in mature fruits (pods) of mustard and meadow herbs of the *Cruciferous* family, the content of mycotoxins is less than in the vegetative part [22-24]. The source of the appearance of mycotoxins in vegetative plants is probably fungi that live in the form of stable associations with the main organism and retain the composition and ratio of components according to species ranks. For extensive communities of endophytic fungi [25], the transmission is known [26] of their metabolites into seeds (through the migration of producers from vegetative parts or vertical transfer in the form of conjugated forms along conducting pathways)

Potentially toxigenic *Alternaria* species are not only among the causative agents of *Alternaria*, which annually leads to significant losses in rape crops [27], but are also capable of semi-parasitic habitation with broad substrate specificity [28] and have recently been identified as dominant among endophytes. in the roots, stems and leaves of rapeseed plants [20]. Given this, it is still premature to conclude about the sources of AOL in the composition of contaminants in spring

oilseed rape seeds under changing external conditions. The possibility of its transmission as one of the metabolites of endophytic fungi under the influence of biotic or abiotic displacements cannot be ruled out. In a single attempt to detect alternariotoxins in the seeds of this crop, none of the six were detected [19].

3. Selective mycotoxicological analysis of seeds from different regions

Region	Mycotoxins, n^+/n, concentration (min-max, μg/kg)	References
Romania	Aflatoxins, 7/7; deoxynivalenol, 7/7; zearalenone, 7/7	[4]
Lithuania	At harvest: aflatoxins, 5/5, (1.0-3.1); ochratoxin A, 5/5 (1.9-7.0); deoxynivalenol, 5/5 (164-183) After 8 months of storage: aflatoxins, 3/5 (2.1-3.3); ochratoxin A, 5/5 (1.3-1.9); deoxynivalenol, 0/5	[15]
Lithuania	Winter barley: deoxynivalenol, 8/8 (153.5-176.5); zearalenone, 12/12 (10.6-25.6); T-2 toxin, 8/8 (8.5-10.2) Spring barley: deoxynivalenol, 6/8 (0-181.0); zearalenone, 12/13 (0-25.10); T-2 toxin, 8/8 (8.2-10.1)	[16]

N o t e. n is the number of samples examined, $n+$ is the number of positive samples containing mycotoxins.

4. A detailed mycotoxicological analysis of rape seeds from different regions

Region	n	Mycotoxins		References
		detected n^+ (concentration, min-max, μg/kg)	not detected	
Spain (Catalonia)	20	Aflatoxin B1 — 1 (0.25)	Aflatoxins B_2, G_1, G_2, alternariol, alternariol monomethyl ether, tenuazonic acid, altertoxins I and II	[19]
China	29	Aaflatoxin B1 — 10 (0.2-0.8); bovericin — 8 (137.6-898.8); fumonisin B_1 — 6 (157.8-474.5); aflatoxin B_2 — 2 (0.6; 1.4)	Aflatoxins G_1, G_2, ochratoxin A, sterigmatocystin, zearalenone, 3- and 15-deoxynivalenol monoacetates, fumonisins B_2, B_3, T-2 toxin, HT-2	[29]

N o t e. n is the number of samples examined, $n+$ is the number of positive samples containing mycotoxins.

According to our data, under difficult harvesting conditions, EA, AOL, MPA, and EMO can be expected among the contaminants of oilseed rape seeds, but such contamination may well be characteristic only of the agrozone in which the observation was carried out. Mycotoxicological evaluation of seeds from other regions, performed on smaller samples, revealed aflatoxins, OA, and fusariotoxins (Tables 3, 4). Thus, from the group of fusariotoxins in Romania in 2002-2004, DON and ZEN were found [4], in Lithuania DON in the crop obtained from experimental crops of winter rapeseed [15]. DON, ZEN and T-2 were identified in 2007-2009 in the seeds of spring and winter rapeseed grown using traditional technology in the Kedai and Panevezys districts of central Lithuania [16]. In China, in seeds obtained from industrial crops in 11 provinces, only fumonisin B_1 and bovericin were found, while ZEN, DON monoacetates, and T-2 were not detected [29]. Apparently, the appearance of fusariotoxins in the seeds was the result of focal damage to plants by complexes of fusarium pathogens that differ in species composition. The capabilities of the analytical approach we used were quite sufficient to confidently determine the degree of contamination of seeds with aflatoxins, OA, and fusariotoxins identified by other researchers (see Tables 3, 4).

In Russia, the oilseed rape seed production and processing had grown rapidly in recent decades, with acreage nearly doubling and seed yields reaching a record 2.4 million tonnes in 2020-2021 [30]. This crop is cultivated in the regions of all federal districts - from the North-Western to the Far East, which differ significantly in soil-climatic and agrotechnical conditions. By-products from the processing of seeds into oil, cake and meal are becoming more and more in demand in feed production, and rapeseed cake biomodification products are considered as promising functional ingredients for the food industry [31]. Under such conditions, the need for a systematic approach to monitoring this raw material in the country is beyond doubt. Unfortunately, regular control of its sanitary quality has not yet been organized.

The results of a point assessment are all the more interesting: a sample of rapeseed cake received from the Kaliningrad region in 2021 did not contain the studied mycotoxins, while that obtained in 2018 from the Krasnodar Territory was contaminated with CPA (50 μg/kg) and EMO (38 μg/kg) (unpublished data of the authors).

The possible accumulation of individual mycotoxins in oilseed rape seeds should not be ignored in agronomic practice, given their negative impact on germination. Effective elimination of seed infection can be ensured by pre-sowing preparation (drageeing, encrustation or disinfection using fungicides), but mycotoxicological control is necessary to reduce the risk of seed contamination.

Thus, on the original (reproductive) seeds of spring and winter crops of oilseed rape, it was shown that, subject to the phytosanitary and technological rules of cultivation and storage, there is no reason to worry about the threat of their contamination with toxins characteristic of fungi of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Myrothecium* and others. All identified cases of seed contamination with mycotoxins were samples collected under a combination of adverse weather conditions or violations of storage rules.

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