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THE COMPLEX OF MYCOTOXINS IN OILSEED RAPE AND TURNIP RAPE DURING SPRING AND SUMMER SEASONS

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Abstract

Oilseed rape and turnip rape crops are widely used to produce seeds and green mass (T.A. Egorova et al., 2015; A.V. Valitov et al., 2018; V.T. Volovik, 2020). The plants are also considered promising siderates that saturate the soil with potassium, phosphorus and nitrogen, and their introduction into crop rotation has a positive effect on grain yields. Recently, the composition and content of mycotoxins were studied in vegetating white mustard and meadow grasses of the *Cruciferous* family with an assessment of seasonal variability and organotropy (A.A. Burkin et al., 2019; A.A. Burkin, G.P. Kononenko, 2022). In this study, it was established for the first time that cyclopiazonic acid. ergot alkaloids, alternariol and emodin are included in the group of the main contaminants of oilseed rape and turnip rape before flowering, as well as data on the expansion of the composition of the mycotoxin complex during budding and the heterogeneous distribution of these substances by plant organs has been received. The aim of this work was mycotoxicological examination of winter turnip rape *Brassica campestris* fr. *biennis* and winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk in the spring-summer growth period — from the rosette phase to the completion of budding, as well as in vegetative and generative organs of plants during flowering and formation of siliques. Vegetating plants were collected from the experimental plots of the Williams Federal Research Center VIC. Winter oilseed rape and turnip rape (sown on September 8, 2020) were collected starting from April 23, 2021, spring rapeseed (sown on May 21, 2021) — from June 25, 2021 weekly. The aboveground parts of whole plants were cut at a height of 3-5 cm from the soil surface, in the phases of flowering and silique formation, the plants were divided into leaves, stems, flowers and siliques. After drying and grinding in a laboratory mill, 349 samples were analyzed. The content of T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), fumonisins of group B (FUM), ergot alkaloids (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 a unified methodology (GOST 31653-2012. Feed. Method of enzyme immunoassay of mycotoxins. Moscow, 2012) using a panel of 15 certified commercial and research enzyme immunoassay systems. The ground samples were extracted with a mixture of acetonitrile and water, 84:16 v/v, at 10 ml per 1 g sample. Indirect competitive enzyme-linked immunosorbent assay (ELISA) was performed after tenfold dilution of extracts with phosphate-salt buffer solution (pH 7.4) with Tween 20. In the entire sample of samples, 14 analytes out of 15 were detected (no POA was found). In winter crops in the rosette-stemming phases, EA, AOL, CPA and EMO were detected in part of the samples with the values located near the limits of the method definition, in the budding phase, an increase in the accumulation of EA, AOL, CPA was observed with cases of detection of EMO, AB_1, STE, OA, MPA and the appearance of fusariotoxins ZEN, FUM. Spring oilseed rape was less contaminated than winter form. During flowering and maturation of siliques, plants showed common patterns of the distribution of mycotoxins by organs, i,e., a greater accumulation in leaves compared to stems and a decrease in the content in ripening siliques. In the flowers of all crops, frequent contamination of MPA was detected, and, as a rule, in combination with EMO, and mycotoxins were

found in winter crops that were absent during the initial growth period (CIT, PR, T-2, and DON). The possibility is discussed of participation of potentially toxigenic micromycetes of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, *Mucor* in plant contamination.

Keywords: winter turnip rape, winter oilseed rape, spring oilseed rape, mycotoxins, enzyme immunoassay

Oilseeds turnip rape *Brassica campestris* fr. *biennis* and winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk are universal forage crops. In addition to waste from seed processing (cake and meal), their herbage and silage are widely used [1]. The intensive rate of crop formation, good regrowth after mowing in the early stages and the possibility of sowing every 10-15 days, can provide a continuous green conveyor [2]. Both species are also important for agrotechnical practice. As green manure, they enrich the soil with potassium, phosphorus and nitrogen and have a positive effect on grain yields in crop rotation. Almost all types of soils and zones are suitable for these crops; green mass can be harvested from early spring to late autumn, until the snow cover is formed [3, 4]. Of the variety of economically valuable cruciferous crops, the nature of mycotoxin contamination has been studied only for white mustard [5]. For vegetative plants of turnip rape and oilseed rape, such an assessment was not carried out.

In recent decades, significant progress has been made in the study of biodiversity and the functional role of microscopic fungi living inside plants [6, 7]. Information about the phylogenetic position, genetic potential, and metabolic capabilities of these organisms is important for accumulating information about their participation in the processes of plant development and adaptation [8, 9]. The study of low molecular weight metabolites the appearance of which is associated with associated toxigenic fungi, in representatives of the *Cruciferous* family began quite recently. For meadow grasses of 13 genera, these investigations revealed composition of mycotoxins and the features of seasonal dynamics and distribution over vegetative and generative organs [10]. The continuation of these works on cultivated plants is of particular interest because of their stable adaptation to the climatic conditions of areas of long-term cultivation and the formation of winter or spring forms.

This paper is the first to report that cyclopiazonic acid, ergoalkaloids, alternariol, and emodin are among the main contaminants of turnip rape and oilseed rape before flowering, and there is an expansion of the mycotoxin complex during budding and the heterogeneous distribution of these substances over plant organs.

The aim of this work was mycotoxicological examination of winter turnip rape, winter oilseed rape, and spring oilseed rape in the spring-summer period of growth, from the rosette stage to the completion of budding, and the vegetative and generative organs of these plants at flowering and pod formation.

Materials and methods. The vegetative plants of the winter rape *Brassica campestris* fr. *biennis*, winter and spring rapeseed *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk cultivars were grown at the experimental plots of the Williams FSC VIK (Moscow Province).

Winter turnip rape and oilseed rape plants sown on September 8, 2020 were cut weekly starting from April 23, 2021, spring oilseed rape sown on May 21, 2021, from June 25, 2021. Plants were cut at 3-5 cm above soil surface; the plants cut at flowering and fruit formation were divided into leaves, stems, flowers, and pods. After drying and grinding in a laboratory mill, 349 samples were analyzed.

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). 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 quantitative 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 rg/kg (PR).

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

Results. In 349 aerial parts of turnip rape and oilseed rape plants, and in vegetative and generative organs of these plants, 14 out of 15 mycotoxins were detected at flowering and maturation, although with various frequencies and often sporadically at a basal concentrations). ROA was not detected.

1. Mycotoxin occurance ($n+$) **and accumulation in winter turnip rape *Brassica campestris* fr. *biennis*, winter and spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk of different varieties at the rosette—stem extention** (1) **and flowering** (2) **stages** (Moscow Province, 2021)

Mycotoxin	Winter turnip rape *B. campestris* fr. *biennis*		Winter oilseed rape *Brassica napus* L. ssp. *oleifera*		Spring oilseed rape *Brassica napus* L. ssp. *oleifera*
	1 (n = 15)	2 (n = 6)	1 (n = 30)	2 (n = 24)	2 (n = 8)
ZEN	–	5	–	2	–
		10-22-26		19, 24	
FUM	–	4	–	2	–
		305-325-340		315, 390	
EA	4	6	9	24	7
	4-7-10	15-21-30	2-3-6	12-32-84	5-19-33
AOL	1	6	3	8	4
	30	30-25-46	16-20-25	26-32-37	(16-19-21)
AB_1	–	2	1	1	–
		1, 1	3	1	
STE	–	2	–	1	–
		10, 20		9	
CPA	3	6	15	24	8
	135-150-170	190-270-400	89-120-160	160-360-980	83-135-200
EMO	3	2	–	4	1
	28-31-33	30, 40		32-34-37	38
OA	–	1	–	1	–
		9		9	
MPA	–	–	–	1	–
				33	

N o t e. ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB_1 — aflatoxin B_1, STE —sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin, OA — ochratoxin A, MPA — mycophenolic acid; n is the number of studied samples. The top figure in the rows is the number of positive samples ($n+$) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

At the beginning of spring regrowth, in the rosette—stem extension stages, EA, AOL, and CPA were detected in both winter crops, and EMO in oilseed rape but only in some samples and in an amount within the detection limits of the method. AB_1 was detected in one oilseed rape sample (Table 1). The onset of the budding stage led to the appearance of fusariotoxins ZEN, FUM and an increase in the EA and CPA accumulation, these toxins were detected in all samples. The average levels of mycotoxin accumulation in turnip rape and oilseed rape were comparable, 21 and 32 μg/kg for EA, 270 and 360 μg/kg for CPA, EMO, AB_1, STE, OA were found rarely, MPA in one of the samples of winter oilseed rape.

AOL was produce in all turnip rapeseed samples (average content of 25 μg/kg) and only in 8 out of 24 oilseed rape samples in comparable amounts. The unequal increase in AOL contamnation in winter turnip rape and oilseed rape

rapeseed during stage changes could be due to differences in the species composition of *Alternaria* fungi. Unfortunately, the available information on this issue is still limited. Isolates of the potentially toxigenic endophytic species *A. alternata* were detected in the stems and leaves of oilseed rape [11]. M.J. Kelman et al. [12] found that in the Canadian population of *Alternaria* colonized oilseed rape plants, the proportion of AOL producing species is small. Representatives of the *A. infectoria* group that do not form this toxin are much more common.

Spring oilseed rape at budding stage was less contaminated than winter turnip rape and oilseed rape (see Table 1). Thus, the accumulation of EA, AOL, and EMO here did not exceed 50 μg/kg, CPA 200 μg/kg, and other toxins were absent. In contrast, 10 out of 15 mycotoxins were found in both winter crops. It is possible that the number of detected mycotoxins increased relative to spring rapeseed was the result of a long spring-spring period of growth, during which a larger part of toxigenic fungi could accumulate mass.

In all examined plants, along with AOL, CPA and EA were typical contaminants. The same mycotoxins dominated in vegetative white mustard and meadow grasses [5, 10]. Apparently, cruciferous communities of associated fungi always contain micromycetes capable of their biosynthesis and begin to function actively from the very beginning of growth.

Any participation of endophytic fungi in the biosynthesis of mycotoxins should be confirmed by the identification of potentially toxigenic species in the deep mycobiota after surface disinfection of tissues. Data which would allow us to make reasonable assumptions about the sources of the appearance of mycotoxins that we found in cruciferous plants are very little. Thus, *Aspergillus flavipes* was found in the stems of vegetative oilseed rape plants, and *Fusarium proliferatum* was found in the leaves [11]. For *A. flavipes* and related species, the ability to biosynthesize STE has been described [13], *F. proliferatum* produces FUM [14]. Unfortunately, so far the interest of researchers in cruciferous endophytes is mainly associated with the search for biological means of protecting these plants from pathogens of fungal diseases [11, 15-17].

The production of CPA and EA is known for micromycetes of many taxa [18]. Some species belong to the endophyte community, for example, *Aspergillus fumigatus* [19], *Penicillium chrysogenum*, *P. commune*, *Mucor hiemalis* [20]. Among the endophyte-dwelling fungi, *A. versicolor* [21], *P. chrysogenum* [20], and *P. brevicompactum* [7] synthesized STE [22], EMO and MPA derivatives [23] were also identified. Detection of OA in turnip rape and oilseed rape during budding may be associated with fungi *P. verrucosum* var. *cyclopium* and *P. chrysogenum* which were previously found in oilseed rape seeds and produced this toxin [24]. In addition to the potentially toxigenic species already known, one should consider the role of other micromycetes, which, as recently found out, possess the corresponding gene clusters [25], and the extensive associations of non-cultured fungi, the presence of which in plant microbiomes has been confirmed by molecular methods.

From the beginning of flowering to the maturation of rapeseed and rapeseed plants, we analyzed the content of mycotoxins in various organs. i.e., stems, leaves, flowers and pods (Tables 2-4). During this period, all plants showed a greater accumulation of mycotoxins in leaves compared to stems and a decrease in the content in ripening pods.

The same patterns were previously noted in white mustard seed and wild herbs [5, 10], which obviously indicates the general directions of the reorganization of their internal microbiome, which are still unknown. Extremely low contamination of ripening pods, especially in spring oilseed rape, is consistent with the absence of mycotoxins in the seeds of this plant (unpublished data of the authors).

2. Mycotoxin occurrence (n+) and accumulation in various organs of winter turnip rape *Brassica campestris* fr. *biennis* plants at flowering, fruit formation and ripening stages (Moscow Province, 2021)

Mycotoxin	Stems (n = 21)	Leaves (n = 11)	Flowers (n = 5)	Green pods (n = 15)	Yellow pods (n = 10)
T-2	–	–	–	–	–
DON	–	–	4 79-82-89	–	–
ZEN	–	–	4 12-13-17	–	–
FUM	–	–	2 105, 125	–	–
EA	18 6-19-40	11 10-15-26	4 10-13-20	15 5-19-42	3 6-13-25
AOL	4 26-32-48	4 18-28-33	5 30-4-49	–	1 54
AB1	–	–	3 1-1-2	–	–
STE	–	–	–	–	–
CPA	18 63-145-350	11 100-195-295	5 155-185-240	13 79-115-235	1 50
EMO	–	4 38-47-58	3 32-34-39	1 31	5 30-42-59
OA	–	–	1 8	–	–
CIT	–	–	2 16, 18	–	–
MPA	–	–	3 40-41-42	1 40	3 13-16-21
PR	–	–	–	–	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin , OA — ochratoxin A, MPA — mycophenolic acid, PR — PR-toxin; n is the number of samples examined, the top figure in the rows is the number of positive samples (n+) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

3. Mycotoxin occurrence (n+) and accumulation in various organs of winter oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk plants at flowering, fruit formation and ripening stages (Moscow Province, 2021)

Mycotoxin	Stems (n = 36)	Leaves (n = 25)	Flowers (n = 16)	Green pods (n = 27)	Yellow pods (n = 28)
T-2	–	–	1 6	–	–
DON	–	–	8 83-105-130	–	–
ZEN	–	–	13 9-16-24	–	–
FUM	–	–	1 100	–	–
EA	31 4-13-30	25 8-83-710	16 6-10-16	27 3-21-63	10 4-10-20
AOL	3 21-23-26	19 17-36-50	16 30-42-56	6 12-24-30	1 24
AB1	–	–	10 1-1-2	–	–
STE	–	1 18	–	–	–
CPA	25 54-130-245	22 105-205-415	15 100-145-200	22 100-150-245	3 79-87-91
EMO	3 30-31-32	4 31-33-39	10 31-37-48	2 (30, 30)	–
OA	–	3 8	3 9-10-10	–	–
CIT	–	1 16	1 16	–	–
MPA	–	1 62	12 30-37-52	3 32-37-40	–
PR	–	–	4 320-380-400	–	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin,

OA — ochratoxin A, MPA — mycophenolic acid, PR — PR-toxin; *n* is the number of samples examined, the top figure in the rows is the number of positive samples (*n*+) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

4. Mycotoxin occurrence (*n*+) and accumulation in various organs of spring oilseed rape *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk plants at flowering, fruit formation and ripening stages (Moscow Province, 2021)

Mycotoxin	Stems (*n* = 19)	Leaves (*n* = 12)	Flowers (*n* = 5)	Green pods (*n* = 19)	Yellow pods (*n* = 17)
T-2	–	–	–	–	–
DON	–	–	–	–	–
ZEN	–	–	–	–	–
FUM	–	–	–	–	–
EA	4	10	5	3	1
	3-5-6	4-8-20	4-5-6	4-5-6	5
AOL	–	2	3	–	–
		21, 26	20-24-26		
AB1	–	–	2	–	–
			2, 3		
STE	–	–	1	–	–
			7		
CPA	4	10	4	4	–
	84-160-250	94-255-300	105-185-290	79-195-315	
EMO	2	3	3	1	–
	43, 50	36-45-50	41-44-51	48	
OA	–	–	2	–	–
			6, 7		
CIT	–	–	–	–	–
MPA	–	3	5	5	-
		16-18-20	15-25-38	15-20-29	
PR	–	–	–	–	–

N o t e. T-2 — T-2 toxin, DON — deoxynivalenol, ZEN — zearalenone, FUM — fumonisins, EA — ergoalkaloids, AOL — alternariol, AB1 — aflatoxin B1, STE — sterigmatocystin, CPA — cyclopiazonic acid, EMO — emodin , OA — ochratoxin A, MPA — mycophenolic acid, PR — PR-toxin; *n* is the number of samples examined; *n* is the number of studied samples. The top figure in the rows is the number of positive samples (*n*+) containing mycotoxins in an amount exceeding the lower limit of measurements; under it the contents of the ycotoxin (μg/kg, minimum-average-maximum) are indicated. A dash means that the mycotoxin was not detected.

In flowers of winter plants (see Tables 2, 3), the number of detected metabolites increased. The flowers of winter turnip rapeseed and winter oilseed rape contained fusariotoxins which were not present at the generative phase either in the vegetative organs (leaves, stems) or in the pods. This has been described in sowing and wild-growing annuals, the white mustard and field cabbage [5, 10]. Formally, winter plants are classified as annuals, since the entire development period does not exceed one year, although it begins in autumn, is interrupted by a period of winter dormancy, and resumes in spring. No fusariotoxins were found in the flowers of spring rapeseed (see Table 4). Perhaps this is due to a shortened initial rosette-stem phase and a rapid transition to budding and flowering.

A clear difference in the accumulation of fusariotoxins in flowers in winter and spring forms definitely indicates the unequal involvement of *Fusarium* toxin-forming fungi in ontogeny. In this case, either a directed movement of the pathogen through the tissues or the transfer of the resulting metabolites from remote points of localization of producers can occur. Given the available information, the second way seems to be more realistic. Thus, it has been shown that phytotoxins spread throughout the plant, while pathogens of the genus *Fusarium* ascend the stem only a few centimeters above the soil level or remain in the root collar.

In the flowers of all crops (see Tables 2-4), frequent MPA contamination was detected, as a rule, in combination with EMO, and in winter crops, mycotoxins were found that were absent in the initial period of growth, the CIT, PR, fusariotoxins T-2 and DON. There are indications in the literature that some species of *Penicillium* and *Fusarium* capable of biosynthesis of MPA (*P. brevicompactum*), PR

(*P. chrysogenum*) [23], T-2 (*F. sporotrichioides*), DON (a complex of species related to *F .graminearum*) [26] are present in the internal mycobiota of plants [20, 27]. Information on belonging to the endophytes of the fungus *Aspergillus pseudoglaucus* which is characterized by co-formation of MPA and EMO (unpublished data of the authors), could not be found. The fact that almost all analyzed mycotoxins, except for ROA, were found in the tissues of the plants examined by us indicates the diversity of toxigenic fungi present in the microbiome.

Thus, for winter turnip rape, winter and spring oilseed rape, a weak contamination with mycotoxins was established with the most frequently detected cyclopiazonic acid in amounts of no more than 360 μg/kg, alternariol and emodin at 32 and 34 μg/kg, respectively, ergoalkaloids at 3-32 μg/kg. A greater accumulation of the components of the main complex and the appearance of zearalenone and fumonisins, as well as aflatoxin B_1, sterigmatocystin, ochratoxin A and mycophenolic acid detected in winter crops at budding stage, after the end of the initial period of growth, was also weakly expressed. Therefore, both economically valuable crops belong to the group with a reduced risk of negative effects on animals. At the generative phase, plants of both winter and spring forms showed an increased content of mycotoxins in leaves compared to stems, a weakening of contamination of ripening pods, and an accumulation of mycophenolic acid and emodin in flowers. The appearance of fusariogenic toxins and citrinin was noted only in flowers of winter plants. Signs of similar shifts and differences in the complex of mycotoxins in cruciferous crops of winter and spring types, established for the first time, indicate complex multidirectional processes of involvement of toxin-forming micromycetes in the development of these organisms. The obtained data may be a basis for further study of the mechanisms regulating the cohabitation of plants and associated fungi.

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