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THE FIRST MYCOTOXICOLOGICAL INVESTIGATION OF WHITE MUSTARD (*Sinapis alba* L.)

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Abstract

Crops widely represented on cultivated lands and often found in natural botanical formations have attracted increasing attention of researchers in recent years. This is due not only to their economic importance, but also to the high value of both experimental facilities that allow studying the features of the formation of the diversity of biocenotic connections and ecological equilibria. According to modern concepts, a complex of secondary substances in plants is increasingly seen as a joint product of their associations with microorganism communities, mainly microscopic fungi (**S.** Kusari et al., 2012). Recently, Russian researchers performed the first cycle of studies aimed at a comparative study of the content of mycotoxins in cereals and legumes in industrial crops and in the natural habitat (G.P. Kononenko et al., 2015; A.A. Burkin et al., 2017). In the present work, we obtained first information about the nature of the contamination of cruciferous plants with toxic metabolites of microscopic fungi, revealed for the first time differences in their localization in vegetative and generative organs, as well as changes accompanying the full development cycle. The aim of this work was to study the composition and content of mycotoxins in the white mustard (*Sinapis alba* L.), a cultivated plant of wide application, which also easily populates agricultural land and occurs in natural grass stands. For analysis, we used overground parts of plants and their organs (leaves, stems, flowers, pods) collected in the white mustard monoculture in 2017 during distinct phases of plant development. These phases were i) the beginning of the growing season after the completed formation of plant basic structure, ii) mass flowering, iii) the formation of green pods and iv) full ripening. The mycotoxins determined by the enzyme-linked immunosorbent assay were T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), aflatoxin B_1 (AB_1), sterigmatocystin (STE), roridin A (ROA), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR) and ergot alkaloids (EA). AOL, CPA, and EA were found in the mycotoxin complex of *Sinapis alba* organs during vegetation period, and all other metabolites were absent or detected sporadically. The very moderate accumulation of mycotoxins in this plant is a useful economic property, and previously no such slightly contaminated cultures were detected among examined cereals and legumes. During the vegetation of the mustard, the composition of mycotoxins and the quantitative ratios between them were generally stable, but the content of AOL and CPA decreased as the plant matured. Mass flowering was accompanied by the appearance in the plant of fusariotoxins DAS, DON, FUM, which were not detected in the next phase (pod formation). In experiments with individual organs of *Sinapis alba*, multiple and intense flower contamination with all analyzed mycotoxins, complete absence of fusariotoxins in green and ripe pods, as well as increased levels of AOL accumulation in leaves compared with stems are established for the first time. Possible causes of this phenomenon, the scientific and practical significance of new information on the degree of contamination, seasonal dynamics and accumulation of mycotoxins in this plant, as well as the prospects for further scientific research are discussed.

Keywords: white mustard, *Sinapis alba*, mycotoxins, T-2 toxin, diacetoxyscirpenol, deoxynivalenol, zearalenone, fumonisins, alternariol, aflatoxin B_1, sterigmatocystin, roridin A, cyclopiazonic acid, emodin, ochratoxin A, citrinin, mycophenolic acid, PR toxin, ergot alkaloids, ELISA

In the recent years, a strong focus has been given to the crops that are widely grown as well as widespread naturally. This is due to the crops' economic importance and involvement in ecological research of biocenotic relations and biological balance. Russian researchers have recently completed the first cycle of a comparative study of mycotoxins in cereal and legume grasses in commercial crops and natural habitat [1-3]. The complex of secondary substances in plants is increasingly seen as a joint product of their associations with microbial communities, mainly microscopic fungi [4].

The cultures of the *Cruciferae* (*Brassicaceae*) family represented in agrobiocenoses by a variety of genera and species have not yet been examined in this aspect. Among them, annual plants are the most attractive as, along with high yields and a short growing season, they differ in soil requirements, resistance to drought, lower temperatures, diseases and pests. These plants are used as the green manure due to their active role in the formation of coenoses — in improving soil quality and soil decontamination, in particular, neutralization of heavy metals [5].

White mustard (*Sinapis alba* L.) is an annual cultivated plant that easily populates agricultural land and natural grass stands, is found on waste grounds, along roads, in gardens and orchards. It is cultivated for seeds that are used to produce edible and technical oil; its young greens are used for food; the crops are used for grazing, grass meal, silage, and in early phases before the formation of pods the white mustard's grass stands are mown for livestock feed.

Recent years have been marked by major achievements in the selection of this culture, especially due to the development of a genomic approach in assessing the diversity of germplasm [6-9]. However, despite its versatile use, the data on the secondary metabolites of white mustard is very limited. For instance, acetylcholine was detected in the leaves and stems, and histamine in the seedlings of the white mustard [10]. Its seeds contain B vitamins, phytosterols, unsaturated fatty acids and sinalbin glucoside [11], as well as groups of nitrogen- and sulfur-containing compounds, the glucosinolates [12, 13]. A detailed analysis of the genetic apparatus disclosed the mechanisms of the biosynthesis of erucic acid, glucosinolates, and the phytochelatin, a fragment of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ peptides formed as a result of a reaction involving phytochelatin synthase (EC 2.3.2.15) [14, 15]. No information was found in the available literature about the toxic properties of micromycetes colonizing this culture, nor about the content of fungal metabolites in these plants or formed *in planta* together with endophytic fungi.

In our research, we were the first to study the nature of the contamination of cruciferous plants by toxic metabolites of microscopic fungi. We have revealed differences in their localization in vegetative and generative organs, as well as changes along the entire development cycle.

Our research focused on the composition and content of toxic metabolites of microscopic fungi in white mustard and their distribution in the plant's organs.

Techniques. The studied samples were mustard plants sown on May 28, 2017 on the trial ground of the Timiryazev Moscow Agrarian Academy. Samples were collected first on days 18 and 25 after sowing as soon as the basic structure of the plant was formed (stage I) and later on days 39 and 41 during the blooming (stage II), on days 53 and 58 during green pods (stage III), and on days 67 and 97 at maturity (stage IV). The above-the-ground parts of the plants were cut at a height of 3-5 cm from the ground. From day 39 after sowing, the plants were sorted into vegetative (stalks, leaves) and generative (flowers, pods) organs, which were later analyzed along the same indicators. The samples were dried in

a shaded ventilated room and then crushed in a laboratory mill. For extraction, a mixture of acetonitrile and water was used (84:16, 10 ml per 1 g of sample). Extracts after a 10-fold dilution with phosphate-saline buffer (pH 7.5) were used for indirect enzyme-linked immunosorbent assay.

The content of mycotoxins T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), alternariol (AOL), aflatoxin B_1 (AB_1), sterigmatocystin (STE), roridin A (ROA), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR toxin (PR), and ergot alkaloids (EA) were determined using certified test systems [16]. The lower limit of measurement corresponded to the 85% antibody binding.

The data are presented as arithmetic mean values (M) and sample mean error (±SEM); statistical processing was done using Microsoft Office Excel 2013 and the Wilcoxon non-parametric total rank test with continuity correction by R version 3.4.3 [17, 18].

1. Mycotoxin incidence (n^+) and content in whole white mustard (*Sinapis alba* L.) plants (μg/kg) **after formation of the basic structure** (stage I), **during bloom** (stage II) **and at green pods** (stage III) (Moscow, 2017)

Mycotoxin	Plant development stage		
	I (n = 8)	II (n = 7)	III (n = 5)
T-2	–	–	–
DAS	–	3 (115±15)	–
DON	–	6 (81±2)	–
ZEN	–	–	–
FUM	–	1 (62)	–
EA	8 (7±1)	5 (3±0)	5 (5±1)
AOL	8 (37±2[a])	7 (43±2[a])	5 (20±1[b])
AB_1	–	1 (2)	–
STE	2 (16, 25)	–	1 (20)
ROA	–	–	–
CPA	8 (370±9[a])	7 (190±7[b])	5 (235±8[b])
EMO	–	1 (25)	–
OA	1 (6)	–	1 (5)
CIT	–	–	1 (40)
MPA	4 (22±1)	–	–
PR	–	–	–

Note. T 2 is T-2 toxin, DAS is diacetoxyscirpenol, DON is deoxynivalenol, ZEN is zearalenone, FUM is fumonisins, EA is ergot alkaloids, AOL is alternariol, AB_1 is aflatoxin B_1, STE is sterigmatocystin, ROA is roridin A, CPA is cyclopiazonic acid, EMO is emodin, OA is ochratoxin A, CIT is citrinin, MPA is mycophenolic acid, PR is PR toxin; n is the number of investigated samples; n^+ is the number of positive samples. Stage I is days 18 to 25 after sowing, stage II is days 39 to 41, stage III is days 53 to 58. Specific mycotoxin levels identified in positive samples or M±SEM are given in parentheses. Dashes indicate that no positive samples were found. Values on the same line with different superscript indices (a, b, c) differ significantly at $p < 0.05$.

Results. Three of 16 mycotoxins: CPA (hundreds of micrograms per 1 kg), AOL and EA (1-2 times less quantities) were regularly detected in young plants before blooming (stage I) (Table 1). MPA was detected only in half of the samples, and CTE and OA in just a few. Such non-uniform detection of substances may have been due to the fact that their concentrations were near the sensitivity threshold of the method or were outside the measurement range.

At stage II (blooming), along with CPA, AOL and EA, we also detected DAS, DON and FUM, and AB_1 and EMO among the mycotoxins with a basal level in single samples. Interestingly, STE, OA and MPA that are rarely detected at the beginning of the growing season were not found in the flowering plants. DAS was dominant among fusariotoxins, while the frequency of DON and FUM were a lot less. A similar relationship with the predominance of DAS was noted earlier in other objects, but its cause is still unclear. In plants with already formed pods (stage III), the same three mycotoxins were present, the CPA, AOL and EA, with the rare detection of CTE, OA and CIT. Statistical processing of the results showed a significant ($p < 0.05$) decrease in the content of AOL and CPA in the ground parts of the mustard during the growing season, but at different times (Table 1). The CPA concentration decreased during flowering and remained the same afterwards; the AOL concentration decreased at a later stage of the formation of pods. The same trend was earlier revealed with the CPA concentration in wild-growing cereal grasses by the end of their vegetation in August—September [1].

The green mass of the mustard is generally characterized by a moderate accumulation of mycotoxins, which is considered a useful economic trait. Among the significant diversity of the studied legume grasses, crops that would be so weakly contaminated have not yet been identified [19]. This can be considered as a manifestation of resistance to fungal diseases, which, along with high yields, soil tolerance, drought tolerance and a short vegetation period, serve as the criteria for assessing the viability of annual oil-bearing crops of wide application. Apparently, the accumulation of mycotoxins is the result of genetically-derived plant relationships with microscopic fungi, which determine the internal mycobiota and the specific profile of secondary metabolites.

The presence of three contaminants in all the examined samples probably indicates that they were caused by endophytic fungi. Given the low accumulation of AOL and EA, there were no highly active producers among those fungi or their habitat conditions did not contribute to intense metabolic reactions. Significant accumulation of CPA, which may be associated with infection with certain *Aspergillus* species [20], is not characteristic of the mustard but it was found in other plants, as often and in larger concentrations [19]. It cannot be ruled out that those metabolites were products of associated biosynthesis involving fungi and plants.

2. Mycotoxin incidence (n^+) and content in whole white mustard (*Sinapis alba* L.) plants (μg/kg) **at massive blooming** (day 39 and day 41, stage II) (Moscow, 2017)

Mycotoxin	Organs		
	stems (n = 4)	leaves (n = 4)	flowers (n = 4)
T-2	–	–	4 (2.8±0.3)
DAS	–	–	4 (365±8)
DON	–	–	4 (170±10)
ZEN	–	–	4 (28±2)
FUM	–	–	4 (100±3)
EA	3 (6±2)	3 (4±2)	4 (5.8±0.8)
AOL	4 (18±1[a])	4 (39±5[b])	4 (77±8[c])
AB_1	–	–	4 (3.3±0.3)
STE	–	2 (14. 14)	4 (26±3)
ROA	–	–	–
CPA	4 (40±8[a])	4 (250±29[b])	4 (455±61[c])
EMO	–	–	4 (45±7)
OA	–	–	2 (8.8)
CIT	–	–	4 (39±2)
MPA	–	–	4 (33±6)
PR	–	2 (100. 115)	4 (335±42)

N o t e. T 2 is T-2 toxin, DAS is diacetoxyscirpenol, DON is deoxynivalenol, ZEN is zearalenone, FUM is fumonisins, EA is ergot alkaloids, AOL is alternariol, AB_1 is aflatoxin B_1, STE is sterigmatocystin, ROA is roridin A, CPA is cyclopiazonic acid, EMO is emodin, OA is ochratoxin A, CIT is citrinin, MPA is mycophenolic acid, PR is PR toxin; n is the number of investigated samples; n^+ is the number of positive samples. Specific mycotoxin levels identified in positive samples or M±SEM are given in parentheses. Dashes indicate that no positive samples were found. Values on the same line with different superscripts (a, b, c) differ significantly at $p < 0.05$; for differences in the AOL and CPA concentrations in the plant's organs the p-value was 0.02857.

Most pathogens of cruciferous fungal diseases are not among the producers of the analyzed substances. Nevertheless, the detection of a relatively large group of background mycotoxins in said plants may be associated with the toxigenic species of the *Penicillium*, *Aspergillus* genera from the so-called secondary fungi associated with the pathogen complex. Among the few harmful diseases of the cruciferous which may cause the accumulation of mycotoxins, alternariosis should be noted along with fusariosis. According to Russian researchers, 9 pathogens of pheodictiosporic hyphomycetales were found, including 5 species of the genus *Alternaria* (including *A. tenuissima*), as well as the fungi belonging to the *A. infectoria* complex [21]. The participation of *Alternaria* toxigenic small spore species can lead to the AOL accumulation in the plant [22, 23), however, judging by the low concentration of this metabolite without evidence of obvious variation during the vegetation, its presence is either not associated with pathogenic species or the activity of the AOL producers is limited by the physiological characteristics of the culture.

To get an idea of the nature of the distribution of mycotoxins in the white mustard and the dynamics of their concentration during the plant's devel-

opment, we investigated the vegetative and generative organs in different periods (Tables 2, 3). In the flowering phase (see Table 2), the stems and leaves retained the signs characteristic of young plants of the 1st collection, except that half of the leaf samples contained PR. Concentrations of AOL and CPA in the leaves significantly ($p < 0.05$) exceeded the ones observed in the stems. The mustard flowers had a very peculiar profile of mycotoxins: they contained all substances except ROA, while DAS, CPA and PR were detected in significant quantities (hundreds of micrograms per 1 kg). It is important to note that we could measure the concentrations of MPA, STE, AB_1, EMO and PR, which were previously assigned to the group of background mycotoxins, i.e. they fell into the scope of definition. Among the newly discovered were CIT as well as the T-2 and ZEN fusariotoxins. Obviously, the expanded composition of mycotoxins in the green mass of mustard that was noted in the flowering phase was mainly due to the contribution of flowers with the partial participation of stems and leaves.

The appearance of fusariotoxins in the flowers may have been due to infection with *Fusarium* fungi along with the complex of toxigenic species. Along with trichothecenes T-2 and DAS characteristic of the fungi *F. sporotrichioides* and *F. langsethiae*, we identified DON and ZEN characteristic of *F. graminearum*, and FUM whose main producers are considered to be *F. verticillioides* and *F. proliferatum* [24]. The weak accumulation of T-2, DON, ZEN, and FUM, as well as the absence of fusariotoxins in the leaves and stems could be due to a short flowering period and a rapid fall of flowers. However, an unusually high accumulation of DAS in the mustard flowers (an average of 365 μg/kg), which was twice larger than the amount of T-2, has not been explained so far. Given the multiple contamination of flowering plants when grown for food, earlier mowing is advisable.

3. Mycotoxin incidence (n^+) and content in whole white mustard (*Sinapis alba* L.) plants (μg/kg) **at phases of green pods** (day 53 and day 58, stage III) **and after pod ripening** (day 67 and day 97, stage IV)

Mycotoxin	Stage III			Stage IV	
	stems (n = 7)	leaves (n = 3)	pods (n = 8)	stems (n = 18)	pods (n = 19)
T-2	–	–	–	–	–
DAS	–	–	–	–	–
DON	–	2 (83, 100)	–	–	–
ZEN	–	–	–	–	3 (28±2)
FUM	–	–	–	–	–
EA	6 (10±2)	2 (2, 4)	6 (3±1)	–	1 (2)
AOL	7 (25±5[a])	3 (68±16[b])	8 (30±4[a])	7 (69±24)	6 (40±5)
AB_1	2 (2, 2)	3 (3±1)	3 (2±0)	–	–
STE	2 (15, 19)	–	2 (15, 16)	1 (13)	5 (14±2)
ROA	–	–	–	–	–
CPA	7 (205±37[a])	3 (220±91[a])	8 (275±31[a])	–	18 (140±7[b])
EMO	–	3 (37±6)	3 (22±2)	2 (25, 28)	2 (21, 26)
OA	–	–	1 (6)	–	1 (5)
CIT	–	1 (40)	–	–	1 (16)
MPA	–	1 (26)	1 (26)	–	–
PR	–	–	–	–	–

Note. T 2 is T-2 toxin, DAS is diacetoxyscirpenol, DON is deoxynivalenol, ZEN is zearalenone, FUM is fumonisins, EA is ergot alkaloids, AOL is alternariol, AB_1 is aflatoxin B_1, STE is sterigmatocystin, ROA is roridin A, CPA is cyclopiazonic acid, EMO is emodin, OA is ochratoxin A, CIT is citrinin, MPA is mycophenolic acid, PR is PR toxin; n is the number of investigated samples; n^+ is the number of positive samples.. Specific mycotoxin levels identified in positive samples or M±SEM are given in parentheses. Dashes indicate that no positive samples were found. Values in the same line with different superscript indices (a, b) differ significantly at $p < 0.05$.

At the third stage, AB_1 and STE were detected in the stems, along with the previously found EA, AOL and CPA. The composition of the analyzed substances turned out to be wider in the leaves and, in addition to EA, AOL, CPA and AB_1, we detected EMO, CIT and MPA; from among the fusariotoxins, only DON was found in some samples (see Table 3). In green pods, the complex of

mycotoxins in the total number of detected substances coincided with their composition in the leaves, with a few differences in the minor components, i.e. STE and OA were detected instead of DON and CIT.

The AOL concentration in the pods did not differ from the stems, but it was lower than in the leaves, and the CPA concentration remained the same as in the leaves and stems. Rare or absent fusariotoxins in vegetative organs (stems, leaves) and pods indicate that there is something precluding their spread throughout the plant, which may be due to the short flowering period and the rapid fall of flowers. At this stage of development, the AOL concentration in the leaves was significantly higher ($p < 0.05$) than in the stems and pods, with no differences with respect to CPA.

The contamination of the plant generally decreased at stage IV, what followed from sporadic or absent mycotoxins in the stems and ripe pods. AOL was found in less than half of the samples, STE and EMO – only in rare cases. Some samples of the pods carried ZEN, OA and CIT in small quantities; CPA and EA, which were found in the green plants, were not detected in the stems. Nevertheless, we could measure the CPA concentration in all samples of the yellowed ripe pods, except one. Its concentration significantly decreased compared to the green pods ($p = 0.000219$) (see Table 3). It is possible that such a sharp decrease in the concentration of this metabolite is associated with the suppression of the functions of endophytic fungi or the completion of the life cycle of the plant.

Interestingly, the first experiments with the sunflower and leguminous plants (red clover, white clover, spring vetch, sweet clover, galega orientalis, lupinus polyphyllus), divided into leaves, stems and flowers, have also revealed regular differences that are yet to be assessed. The uneven distribution of mycotoxins in plants can be the result of migration of both the metabolites and toxigenic fungi, as well as the result of physiological and metabolic contacts within the fungal community and with the host organism. Interpretation of the obtained results from the point of view of the role of pathogenic and endophytic fungi in the symbiosis, with the differentiation between the groups of basic (constitutive) and induced mutualism, will be the next important step in understanding the mechanisms of interaction between higher plants and microscopic fungi.

The mature mustard pods were generally very weakly contaminated with mycotoxins. This fact is of great practical importance, since the mustard's seeds have been lately considered as a unique and promising source of physiologically active substances. For instance, it has been revealed that the mustard seeds contain a complex of compounds that inhibit the formation of tumors as well as have antibacterial and antioxidant effects [25]. It has been experimentally proven that the products of the hydrolysis of glucosinolates, 4-methylsulfanyl-3-butenyl isothiocyanate in particular, have chemoprotective properties and can suppress carcinogenesis processes [26]. Waste from seed squeezing or extracting is used in feeding animals, although it is recommended to use mustard cakes and residues with care because of the simple nitriles that are formed during the specific enzymatic hydrolysis of different glucosinolates [27]. It is equally important to study the contamination of this animal food with mycotoxins because the example of other oilseeds, the sunflower, has revealed significant differences in the composition of toxicants in the seeds vs. products of its processing and storage [28].

In our experiments, the white mustard seed was characterized by a tendency to decrease the AOL and CPA concentration as the plants mature. Considering that seasonal mycotoxin dynamics in leguminous grasses, on the con-

trary, revealed that their concentration remained the same or grew [19], it seems important to undertake mycotoxicological examination of *Sinapis alba* from mixed naturally formed communities.

In the future, such projects are advisable for other cruciferous plants that are considered promising for introduction in feed production and farming, such as oil radish (*Raphanus sativus* var. *oleifera* Metzg.), redberry (*Camelina* sp.), abyssian cramba (*Crambe abyssinica* Hochst.), winter rape (*Brassica napus* L. ssp. *oleifera* Metzg.), winter rape (*Brassica rapa* L. ssp. *oleifera automnalis*) and others, as well as the wild-growing members of this family.

Therefore, a complex of toxic metabolites of mycogenic origin in the white mustard under monoculture conditions is mainly represented by alternariol, cyclopiazonoic acid, and ergot alkaloids, and its composition remains stable at all phases of the plant development. For alternariol and cyclopiazonic acid, preferential localization was revealed in the leaves vs. stems, while the flowers have a multiple contamination, with an increased accumulation of metabolites and the presence of fusariotoxins. The concentration of cyclopiazonoic acid decreases in the plant during the blooming, while alternariol decreases during the green pods phase. This new data and the identified patterns are very important for continuing the fundamental research of the biological role of fungal metabolites in plants, as well as for finding appropriate measures to prevent intoxication due to the contamination of food, feed and the environment with mycotoxins.

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