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TOXIN-PRODUCING SMALL-SPORE *Alternaria* SPECIES FROM OAT GRAIN CONTAMINATED WITH ALTERNARIOL

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Abstract

For many years, the problem of grain infestation with toxin-forming fungi *Alternaria* has been under the close attention (S.M. Tralamazza et al., 2018). Extensive studies have been carried out on wheat (M.T. Amatulli et al., 2013; M.E. Müller, U. Korn, 2013) and barley (V. Sanchis et al., 1993; T.T.T. Nguyen et al., 2018). The grain of oats has been studied much less, and it is still unclear which species of fungi of this genus and to what extent are responsible for the accumulation of the toxin alternariol (AOL). In our country, data have been obtained on the infection with *Alternaria* fungi of oat grains from a number of regions (O.P. Gavrilova et al., 2016; Yu.I. Vargach et al., 2019), as well as grain samples of varieties and lines from the VIR collection detected in field tests (A.S. Orina et al., 2017), however, the frequency of occurrence of the toxin was estimated for several regional lots in total (A.A. Burkin et al., 2015; G.P. Kononenko et al., 2020). In this study, it was established for the first time that the species *A. tenuissima* (Nees et T. Nees:Fries) Wiltshire, which is known as an active AOL producer, and to a lesser extent representatives of *A. arborescens* E.G. Simmons and the '*A. infectoria'* complex can participate in the contamination of oat grains. The aim of the work was to study the species affiliation and toxin-forming ability of *Alternaria* fungi isolated from oat grain with natural AOL contamination. The object of the study was a sample obtained in October 2020 from an agricultural enterprise of the Moscow Province (Odintsovo District) containing AOL in the amount of 630 ppb. Isolation of pure fungal cultures was carried out after surface sterilization of grain and its sowing on Chapek-Dox agar containing bile and antibiotics. Color, structure, and growth rate of colonies were described on yeast extract sucrose agar (YES) and potato-carrot (PCA) on day 8. To assess their toxin formation, oat grain and a panel of four mycological media were used — PCA, malt extract agar (MEA), hay infusion agar (HAY) and an analog of vegetable agar (V-8). After cultivation (7 days, 25 °C, without lighting) and extraction of biomass samples with a mixture of acetonitrile and water in a volume ratio of 84:16, AOL was determined by ELISA test (A.A. Burkin, G.P. Kononenko, 2011) with a detection limit of 0.01 μg/g. In the subepidermal mycobiota of the studied sample, representatives of the genus *Alternaria* were predominant, the degree of infection was 36.0 %, and they were accompanied by fungi *Fusarium* spp. (14.7 %) and *Epicoccum* spp. (2.7 %). After carrying out mycological procedures for the isolation and identification of *Alternaria* cultures, they were assigned to the species *A. tenuissima* (Nees et T. Nees:Fries) Wiltshire (7 strains), *A. arborescens* E.G. Simmons (2 strains) and to the species complex '*A. infectoria*' (4 isolates). On the grain substrate, all strains of *A. tenuissima*, *A. arborescens* and three isolates of '*A. infectoria*' produced AOL in amounts of 370, 5 and 0.8 μg/g. When testing cultures under the same conditions on agar media, the intensity of AOL accumulation in *A. tenuissima* was highest on HAY and MEA (56 and 23 μg/g), in *A. arborescens* and '*A. infectoria*' — on PCA and MEA. Taking this into account, commercial substrate MEA and oat grains are recommended for in vitro evaluation of the biosynthetic potential of fungi and their involvement in contamination of AOL grains in an expanded format. The cultural and morphological features of several cultures of *A. arborescens* and '*A. infectoria*' and their ability to AOL biosynthesis are discussed in a comparative aspect.

Keywords: oat grain, *Alternaria tenuissima*, *A. arborescens*, '*A. infectoria'*, alternariol, ELISA

Recently, the problem of grain infection by toxin-producing fungi of the genus *Alternaria*, mainly wheat [1-3] and barley [4, 5], has been actively discussed in the scientific literature. Oat grain, which in the coarsely ground form is widely used in feeding dairy cows, sheep, pigs, rabbits, poultry and is dietary indispensable for horses [6)], has been studied much less [7]. Among the toxins of *Alternaria* fungi, experts are particularly concerned about alternariol (AOL), a metabolite of the dibenzo-α-pyrone group for which a genotoxic effect has been confirmed [8, 9].

Signs of intoxication in animals when fed with infected AOL containing oats were reported at the end of the last century [10]. However, further studies used either mycological or toxicological analyses. Thus, in a sample of oats from Greece, potential producers of *A. alternata* were identified, but no search for the toxin was carried out [11], and when AOL was detected in grain from Sweden [12], southern Norway [13], Canada [14], Ireland [15], and Slovenia [16], mycological analysis was not performed.

In our country, the species composition of *Alternaria* fungi was studied on grain from a number of regions [17, 18] and in field tests on breeding varieties and lines from the collection of the Vavilov All-Russian Institute of Plant Growing (VIR Collection) [19, 20], but toxin contamination is reported only for several regional samples [21, 22]. Recently, attempts to compare toxin contamination and the DNA amounts of fungi of the sections *Alternaria* and *Infectoriae* were made on several samples from the Ural region [23] and Western Siberia [24]. The *Alternaria* species that are responsible for the accumulation of this toxin in grain remain unclear.

In the present study, it was established for the first time that the species *Alternaria tenuissima* (Nees et T. Nees:Fries) Wiltshire, known as an active producer, and to a lesser extent representatives of *A. arborescen*s E.G. Simmons and the '*A. infectoria*' complex may be involved in the oat grain contamination.

The purpose of the work is to study the species and toxin-forming ability of *Alternaria* fungi isolated from oat grains naturally contaminated with alternariol.

Materials and methods. The mycological study was conducted on grain of oat (*Avena sativa* L.) variety Yakov, obtained in October 2020 from an agricultural enterprise located in the Moscow Province (Odintsovo District). According to enzyme immunoassay measurement, the grain samples were contaminated by AOL (630 μg/kg) and T-2 toxin (5 μg/kg), other mycotoxins (deoxynivalenol, zearalenone, group B fumonisins, aflatoxin B_1, sterigmatocystin, ochratoxin A, citrinin, cyclopiazonic acid, mycophenolic acid, emodin, ergoalkaloids, roridin A, PR-toxin) were absent.

The seeds were superficially disinfected with a 3% formaldehyde solution for 1.5 min, followed by double treatment with an aqueous ammonia solution prepared by adding 4 ml of a 5% ammonia solution to 1 liter of sterile distilled water. The seeds were then placed in Petri dishes on the surface of Czapek-Dox agar containing bile and antibiotics [25]. After 7 days of culture at 25 °C, *Alternaria* were seeded onto Petri dishes and, after confirming their purity, onto agar slant. The isolated cultures were identified to species using manuals [26, 27]; monoconidial strains were obtained as described previously [28]. Description of the color, structure of colonies, and growth rate of cultures was performed on syeast extract sucrose agar (YES) and potato-carrot agar (PCA) on day 8.

To quickly assess the ability of cultures to produce AOL, grain substrate (oat flakes), PCA, malt extract agar (MEA; Liofilchem®, Italy), hay infusion agar (HAY), analogue of agar V-8 from vegetable juice (Southern Juice Company LLC, Krasnodar Province, Belorechensk, Russia) prepared according to the appropriate recipe [29] were used as growth media. The inoculum (10-day cultures on Czapek-

Dox agar) was placed in triplicate into 10-ml vials with a bottom diameter of apprx. 18 mm, each containing 1.5 ml of agar media or 1.0 g of oat flakes with 1.0 ml water added before sterilization. The vials were closed with cotton-gauze caps and wrapped in a layer of laboratory film (Parafilm "M"® PM-996, Pechiney Plastic Packaging, USA). After incubation in the dark for 7 days at 25 °C, each vial was added with 1.5 or 3.0 ml (for grain substrate) mixture of acetonitrile and water (84:16 v/v) and shaken vigorously at the beginning and end of a stationary 14-hour-long extraction. The extracts were analyzed for AOL using a test system for enzyme immunoassay determination of the toxin [30] with its detectable limit of 0.01 μg/g.

The data was processed using descriptive statistics in Microsoft Excel 2013, the results were expressed as arithmetic mean values (*M*) with standard error of mean (±SEM).

Results. Representatives of the genus *Alternaria* predominated in the subepidermal mycobiota of the studied sample. The infection incidence rate was 36.0%, additionally, *Fusarium* spp. (14.7%) and *Epicoccum* spp. (2.7%) were detected. Intensive infection with Alternaria fungi was quite consistent with significant grain contamination with AOL (630 μg/kg), and the detection of *Fusarium* fungi explained the presence of T-2 toxin in grain.

1. Alternariol (AOL) **production by representatives of the genus *Alternaria* isolated from grain of oat (*Avena sativa* L.) variety Jacob grown on a grain substrate** (oat flakes; 7 days, 25 °C, no light) ($n = 3$, M±SEM)

Strain No.	AOL, μg/g substrate
A. tenuissima	
1	140±30
2	290±70
7	370±70
9	460±90
11	115±20
12	1200±70
15	11±1
A. arborescens	
5	4±1
8	6±1
'*A. infectoria*'	
6	–
13	1.5±0.40
14	0.7±0.15
16	0.2±0.04

N o t e. A dash means that AOL was not detected.

After mycological isolation and identification procedures, *Alternaria* cultures were assigned to the species *A. tenuissima* (Nees et T. Nees:Fries) Wiltshire (7 strains), *A. arborescens* E.G. Simmons (2 strains) and to the species complex '*A. infectoria*' (4 isolates). Previously, for grain from five regions of the North-West region, based on the results of morphological identification, the dominance of *A. tenuissima* was shown with a lower occurrence of *A. arborescens* and '*A. infectoria*' [17]. For 5 grain samples from two regions of the Ural Federal District studied by quantitative PCR, a higher infection was reported for the *Alternaria* section fungi (40.8±5.6%) compared to the *Infectoriae* section (2.0±1.1%) [23]. The same relationship was observed for the sample studied. It should be noted that the traditional assignment of fungi to species levels is still considered quite acceptable, despite the increasing use of molecular technologies for DNA detection, e.g., real-time PCR and quantitative digital PCR [31].

In an experiment with short-term culture of fungi on a grain substrate, all cultures except one ('*A. infectoria*' No. 6) produced AOL (Table 1). For *A. tenuissima* strains, the sample average amount was 370 μg/g, indicating a high potential

for toxin production. In *A. arborescens* and '*A. infectoria*' accumulation was significantly less, 5 and 1 μg/g, respectively (see Table 1).

The data obtained for the *Alternaria* isolates from the sample clearly indicated that A. tenuissima was the predominant contributor to the toxin contamination, with the joint participation of *A. arborescens* and '*A. infectoria*'. Of course, this result cannot be extrapolated to the situation as a whole. To establish the composition of the producers responsible for the contamination of oat grain, an extensive survey of the population of fungi associated with this biological object is necessary, organized according to the "one isolate—one sample" principle. When using this strategy for a large set of 58 samples, it was established that *A. alternata*, *A. tenuissima* and *A. arborescens* are involved in the contamination of feed grain products and shown that mycological media may be used for testing fungi. Moreover, from this work, the observations of correspondence between toxin production and color, colony structure, and growth rate of fungi began [28]. This approach was developed in the present study.

Results of testing cultures of A. tenuissima, A. arborescens and 'A. infectoria' for the ability to produce AOL on a panel of four agar media PCA, HAY, MEA, an analogue of V-8, recommended for species identification [26, 27], are submitted in Table 2.

2. Alternariol (AOL) **production by representatives of the genus *Alternaria* isolated from grain of oat (*Avena sativa* L.) variety Jacob grown on mycological agar media** (7 days, 25 °C, no light) (n = 3, M±SEM)

Strain No.	AOL, μg/g substrate			
	PCA	HAY	MEA	analogue V-8
		A. tenuissima		
1	0.06±0.020	2.9±0.50	40±9	0.8±0.05
2	0.9±0.05	77±8	18±2	9±3
7	0.7±0.10	36±8	11±1	5±2
9	0.9±0.30	98±1	21±3	13±3
11	15±5	16±1	38±11	2.0±0.20
12	2.5±0.30	107±27	7±1	16±5
15	–	–	–	–
		A. arborescens		
5	0.4±0.20	0.09±0.020	0.2±0.02	0.1±0.02
8	0.1±0.03	–	0.03±0.010	–
		'*A. infectoria*'		
6	–	–	–	–
13	–	–	–	–
14	1.3±0.90	0.08±0.030	0.1±0.02	0.03±0.006
16	0.08±0.020	–	0.03±0.003	–

Note. A dash means that AOL was not detected. For description of media composition, see Materials and methods section.

On all media, 6 *A. tenuissima* strains produced AOL. The accumulation of the toxin varied from 0.06 to 107 μg/g both between strains and on different media. In one strains (No. 15), the toxin was not detected. The unevenness of AOL biosynthesis in *A. tenuissima* was previously reported [28], and this feature was associated with intraspecific differences that could not be detected by morphological features [32]. In general, the intensity of AOL accumulation in *A. tenuissima* turned out to be greatest on HAY and MEA media (56 and 23 μg/g) and was 1-2 orders of magnitude less than on grain (see Table 1). The same reduction from 300 μg/g (on grain) to 26 μg/g (on MEA) was previously reported for the strain *A. tenuissima* Al 392 isolated from oats [33].

Cultures of *A. arborescens* produced much less AOL than *A. tenuissima*. On HAY and V-8, its amount is detectable only in strain No. 8. On PCA and MEA, the amount of toxin varied from 0.03 to 0.4 μg/g (see Table 2) and, as in *A. tenuissima*, was significantly lower compared to that for grain substrate. Previously, in

three strains of *A. arborescens* from wheat grain and sunflower seeds, a more intense accumulation of AOL on MEA was noted, from 3.3 to 36 μg/g [34], in five crops from grain feed and three collection strains from 2 to 79 μg/kg [28]. Unfortunately, due to the small number of isolates available for study, information on the potential for AOL biosynthesis in this species remains insufficient.

Representatives of the species complex '*A. infectoria*' in terms of toxin production on agar media differed in pairs No. 6, No. 13 and No. 14, No. 16, the first two strains did not produce toxin, while in others it was detected. In both isolates, the ability to produce was detected only on PCA and MEA in comparable quantities, 0.08-1.3 and 0.03-0.1 μg/g, respectively.

Compilation of these data shows that of all mycological media tested, commercial MEA media provided a consistent positive metabolic response for *A. tenuissima*, *A. arborescens* and '*A. infectoria*' and, therefore, can be recommended, along with the grain substrate, for in vitro assessment of their biosynthetic potential in an expanded format.

According to morphological characteristics, all strains of *A. tenuissima* were typical. They had unbranched chains consisting of 5-10 subulate conidia with an elongated neck, dense velvety colonies of dark gray color with a black reverse side, and a moderate growth rate on PCA and YES. No peculiarities in color, colony structure, or growth rate were identified in the only non-producing strain No. 15. Considering the detection of AOL during its culturing on a grain substrate, albeit in the smallest quantity (see Table 1), it is possible to assume its biosynthesis on agar media, but in concentrations below 0.01 μg/kg, that is, beyond the detection limit of the method.

Cultural and morphological traits of *A. arborescens* strains with weak sporulation and isolates of '*A. infectoria*' that did not sporulate in out tests are submitted in Table 3.

3. Cultural and morphological traits of *Alternaria arborescens* strains and the species '*A. infectoria*' complex isolated from grain of oat (*Avena sativa* L.) variety Yakov grown for 8 days on two agar media

Strain No.	Structure, color and diameter (d) of colonies	
	PCA	YES
	A. arborescens	
5	Velvety, dark gray	Dense velvety with distinct concentric circles, gray, the reverse side almost black, d = 47 mm
8	Velvety, light gray, dark gray in the center	Dense velvety, gray, the reverse side almost black, d = 39 mm
	'*A. infectoria*'	
6	Loose fluffy, dark gray	Slightly fluffy, pink-white-gray, the reverse side almost black, d = 55 mm
13	Loose, stringy, light gray	Loose fluffy, white-pink, the reverse side gray, d = 39 mm
14	Felt-like, dark gray	Dense, velvety, white-pink with slightly pronounced concentric circles, the reverse side is brownish, d = 40 mm
16	Felt-like, light gray in the center, gray at the edges	Dense, velvety, white-pink, the reverse side is brownish, d = 50 mm

N o t e. For description of media composition and culture conditions, see Materials and methods section.

A. arborescens colonies on PCA and YES practically did not differ in size and appearance, but in strain No. 5, which, unlike No. 8, produced toxin on all four media (see Table 2), there were clearly visible concentric circles on YES. '*A. infectoria*' colonies were also approximately the same in size (with diameters of 39-55 mm), but differed in pairs in density (No. 6, No. 13 and No. 14, No. 16) (see Table 3) and in the ability to form toxins (see Table 2). Isolate No. 6 which differs sharply from all others in the color of the aerial mycelium and the reverse side on YES, did not produce the toxin both on agar media (see Table 2) and a

grain substrate (see Table 1). Its paired isolate No. 13 which did not produce AOL on agar media but synthesized it on grain, had more signs of cultural similarity to producers No. 14 and No. 16. The maximum accumulation of AOL (1.3 μg/g on PCA) was characteristiv of isolate No. 14 forming dense velvety colony with concentric circles (see Table 3).

This work is the first report on the ability of '*A. infectoria*' isolates from oat grain to produce AOL. The production ability of this complex from wheat grain is known, but there is no detailed description of their macromorphological characteristics. For a strain of Russian origin, 2.01 μg/g AOL production was recorded [35], for 12 strains from Italy, it ranged from 0.3 to 20 μg/g with an average value of 4 μg/g [36], for 98% of isolates in Argentina the value ranged from 1.8 to 433.3 μg/g with an average of 62.2 μg/g [37]. The '*A. infectoria*' morphotypes differing in pigmentation was first reported by B. Kosiak et al. [38]. Recently, an '*A. infectoria*' isolate from sunflower seeds atypical for colony structure and increased growth rate was shown to produce from 2 to 220 μg/g AOL on agar media [28]. Continuing the study of toxin production and culture properties of fungi of this systematically complex group remains relevant for clarifying a number of taxonomic aspects [39, 40].

Thus, this paper described correspondences between macromorphological characteristics and the ability to produce alternariol for a number of *Alternaria arborescens* strains and isolates of the '*A. infectoria*' from Yakov oat variety grain. A comparative assessment of alternariol biosynthesis rate by *A. tenuissima*, *A. arborescens* and '*A. infectoria*' on the panel of agar media recommended for species identification shows that commercial malt agar, along with a solid grain substrate, are suitable for advanced toxicological monitoring. The grain contamination with alternariol is mostly due to *A. tenuissima*, and to a lesser extent to *A. arborescens* and the '*A. infectoria*'. A similar complex methodological scheme, including mycological and toxicological analysis, should be applied for furthet detailed assessment of small-spored Alternaria species populations from oat grain.

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