AGRICULTURAL BIOLOGY,
2020, V. 55, № 3, pp. 628-637
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.085.19:636.086.1/.3:632.4

doi: 10.15389/agrobiology.2020.3.628eng
doi: 10.15389/agrobiology.2020.3.628rus

PRODUCTION OF ALTERNARIOL IN THE POPULATIONS OF GRAIN FEED-ASSOCIATED SMALL SPORE *Alternaria* SPECIES

G.P. KONONENKO, E.A. PIRYAZEVA, A.A. BURKIN

All-Russian Research Institute for Veterinary Sanitation, Hygiene and Ecology — Branch of FSC ARRIEV RAS, 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail kononenkogp@mail.ru (✉ corresponding author), piryazeva01@yandex.ru, aaburkin@mail.ru
ORCID:
Kononenko G.P. orcid.org/0000-0002-9144-615X
Burkin A.A. orcid.org/0000-0002-5674-2818
Piryazeva E.A. orcid.org/0000-0001-5443-3213
The authors declare no conflict of interests
Received February 18, 2020

Abstract

Modern science has strong evidence that *Alternaria* fungi pose a serious toxic hazard. *Alternaria* species can grow well on various substrates and in a wide range of temperatures and humidity, occupying different ecological niches in this way, and can produce several types of especially dangerous secondary metabolites (S.M. Tralamazza et al., 2018). The most well-studied *Alternaria* mycotoxin alternariol (AOH), a dibenzo-α-pyron derivative, exhibits high cytotoxicity, genotoxic and mutagenic effects (Z. Mao et al., 2014). However, the ability of *Alternaria* fungi to produce this toxin still remains poorly studied. In Russia, a significant prevalence of small spore *Alternaria* species on grain crops (Ph.B. Gannibal, 2004, 2006; T.Yu. Gagkaeva et al., 2012), and an increase in the frequency of *Alternaria* occurrence and AOH accumulation in grain and feed mixtures (G.P. Kononenko et al., 2019, 2020) have been recently reported. In this article, we first showed that the species *A. tenuissima*, *A. alternata*, and *A. arborescens* can cause AOH contamination of grain feeds. The work aims to investigate AOH production by the grain feed-associated *Alternaria* species. *Alternaria* fungi were isolated from 57 feed samples of different types (wheat, barley, corn and oats, sunflower seeds, wheat bran and mixed feeds). Monoconidial isolates identified by morpho-cultural features as *A. tenuissima* (Nees et T. Nees: Fries) Wiltshire, *A. alternata* (Fr.) Keissl, and *A. arborescens* E.G. Simmons, and another 14 isolates assigned to *A. infectoria* species group were cultured for 7 days at 25 °C on a panel of 4 mycological media, the potato-carrot agar (PCA), hay infusion agar (HAY), malt extract agar (MEA), and vegetable juice agar (V-8). AOH was detected in extracts using a certified commercial kit for indirect competitive enzyme-linked immunosorbent assay (ELISA). Among the isolates belonging to *A. infectoria* species group, 13 were devoid of producing ability while for one of them the accumulation of AOH was observed on all growth media in quantities 2.0±0.2, 14±3, 18±4 and 220±30 μg/g, respectively. Evaluation of the biosynthetic potential of *A. tenuissima* and *A. alternata* showed the highest degree of its realization on MEA growth medium in terms of the total number of producers and the share of highly active and superactive isolates. The total amount of AOH accumulation in these conditions for both species was almost the same and amounted to 73 and 71 μg/g, respectively. *A. arborescens* isolates provided the highest AOH production on V-8, HAY, and MEA media in amounts equal to 106, 64, and 31 μg/g, respectively. The peculiarities of metabolic response of *A. tenuissima*, *A. alternata* and *A. arborescens* species to environmental changes and a rapid method to assess toxigenicity of *Alternaria* fungi during taxonomic identification are discussed.

Keywords: alternariol, *Alternaria tenuissima*, *Alternaria alternata*, *Alternaria arborescens*, *Alternaria infectoria* species group, grain feeds, ELISA

Modern research convincingly evidence that fungi of the genus *Alternaria* pose a serious toxicological hazard [1]. Owing to the possibility of active growth on different substrates in a wide range of temperatures and humidity, these fungi occupy a vast ecological niche and are capable of producing secondary metabolites of several structural types with especially dangerous negative action [2-5]. In the group of dibenzo-α-pyrones, the most known is alternariol (AOL) possessing high cytotoxicity, genotoxic and mutagenic effects [6].

Assessment of the risk of mycotoxin contamination of natural objects

susceptible to infection by microscopic fungi implies a phased implementation of a complex of mycological and mycotoxicological investigations. First, in a representative set of samples that most fully characterizes the object of the survey, the main species are identified, and the data on the toxin-forming ability of the set of isolates are extrapolated to the entire population. Similar projects for grain infected with *Alternaria* fungi has already been undertaken several times for agroecosystems in Latin America [7-9], Europe [10, 11] and Asia [12, 13], but comparison of the results often failed because of the ambiguity of approaches to fungal species discrimination and differences in the experimental testing schemes. In Russia, the potential for the AOL formation by *Alternaria* fungi that infect grain products remains practically unexplored, and only one of the works of German researchers provides data for 24 isolates from grain of one field in the Novosibirsk region [14].

It should be admitted that the difficulties associated with the variety of methods for determining species of *Alternaria* fungi are objective. The taxonomic system of this genus is currently improving [15, 16]. Morphological identification is recognized as not always unambiguous, since some traits can overlap between different species. The development of molecular methods, as well as a polyphase approach using metabolic profile data, is still at the stage of information accumulation [17-19]. In this regard, when assessing biological objects, researchers are increasingly inclined to choose the traditional approach based on a set of morphological characteristics of reproductive structures and sporulation under controlled conditions [20].

The procedure for testing fungi toxin formation in vitro undoubtedly needs to be unified. For this purpose, it is preferable to use homogeneous agar media but not grain substrates, on which the required precision of determination is not always achieved. Short-term 7-day incubation at 23-25 °C on such matrices followed by analysis of metabolites in blocks of mycelial-spore biomass is widely used in chemotaxonomy of the genera *Penicillium* and *Aspergillus* [21, 22].

Here, we for the first time evaluated the ability of *Alternaria* fungi to produce AOL on a panel of four growth media recommended for the identification of the members of this genus. Our findings revealed the species-specific metabolic response to the type of growth medium and, in addition, showed for the first time that three morphological species, the *Alternaria tenuissima*, *A. alternata*, and *A. arborescens* may be involved into grain feed contamination with this toxin.

Our goal was to study the potential for the production of alternariol in populations of small-spore species of the genus *Alternaria* fungi associated with grain feed.

Materials and methods. Primary cultures of *Alternaria* fungi were isolated from 57 samples of seven types of grain feeds, the wheat grain (29 samples), mixed feed (12 samples), barley grain (7 samples), sunflower seeds (6 samples), and wheat bran, corn grain and oats (one sample each). The fungal species was identified by cultural and morphological characteristics as described [23]. Monoconidial cultures of *Alternaria tenuissima* (Nees et T. Nees:Fries) Wiltshire, *A. alternata* (Fr.) Keissl and *A. arborescens* E.G. Simmons derived from conidial suspensions in 0.1% sterile Tween 80. Controlling the number of conidia in one drop with a diameter of 0.4 cm, 3-4 drops were put into sterile Petri dishes filled with a thin layer of melted and cooled Chapek-Dox agar and cultured for 1 day at 23-25 °C. The agar fragments with single germinated conidia, after being examined under a microscope, were put onto a Chapek-Dox agar slant.

A scheme of 1 isolate—1 sample was applied for testing each *Alternaria* species. In total, there were 14 polyconidial isolates of the *A. infectoria* group of species and monoconidial isolates of *A. tenuissima* (23 isolates), *A. alternata* (20

isolates) and *A. arborescens* (8 isolates, including 3 strains isolated from barley, Nos. 157011, 158011, and 529051 of the Yachevsky Laboratory of Mycology and Phytopathology mycological herbarium, All-Russian Research Institute of Plant Protection, St. Petersburg, Russia).

For morpho-cultural characterization of isolates assigned to the group of *A. infectoria* species, yeast extract sucrose agar (YES) was used. Ten-day old cultures in Chapek-Dox agar served as inocula. The growth media were potato-carrot agar (PCA), hay infusion agar (HAY) prepared as per [24], malt extract agar (MEA) (Liofilchem S.r.l., Italy) and vegetable juice agar (V-8) prepared from vegetable juice (LLC South Juice Company, Krasnodar Territory, Belorechensk, Russia) as per [23]. Each inoculum (in 3 replicates) was put into a 10 ml flask (a bottom diameter of about 18 mm) with 1.5 ml of one of the above media. The flasks were closed with cotton-gauze plugs, additionally wrapped with Parafilm M (Merck KGaA, Germany) and incubated in the dark for 7 days at 25 °C; then each flask was added with 1.5 ml of a mixture of acetonitrile and water, a volume ratio of 84:16, and vigorously shaken at the beginning and end of stationary 14-hour extraction. The AOL levels in the extracts were quantified using ELISA test for alternariol [25] with 0.01 μg/g detection limit of the toxin.

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

Results. The recent data of an extensive study of the *Alternaria* species composition in the European Russia indicate that *A. tenuissima* and *A. infectoria* species complex are most common in cereal seeds, while *A. alternata* and *A. arborescens* are much less frequent [26-28]. The results of the first survey of grain fodder definitely indicated that *A. tenuissima* predominates in the mycobiota of wheat and barley grains [29].

In this work, using more than 150 samples of grain feeds (mainly wheat, barley, sunflower seeds and mixed feeds with a high proportion of grain components), we managed to form sets of enough size only for isolates of *A. tenuissima*, *A. alternata* and *A. infectoria* group of species. Isolates identified as *A. arborescens* were found only in 5 samples, that is why the accessions of similar origin were additionally taken. We thought it expedient to test isolates on a panel of media recommended for the species identification procedure, since this approach could be used in the future for the rapid detection of toxin production in these fungi already at the stage of mycological analysis. Each of the 66 strains were grown under identical conditions on PCA, MEA, HAY and V-8 agars. This work is the world's first comparative analysis of toxin production in *A. tenuissima*, *A. alternata*, *A. arborescens* and *A. infectoria* group on PCA, HAY and V-8 growth media.

All *A. tenuissima* isolates produced AOL on MEA, HAY, and V-8, while no toxin was found on PCA in two isolates (Table 1). On all media, fluctuations in the amount of the toxin in isolates were three orders of magnitude. We noted this feature earlier, and it was even more pronounced. E.g. the AOL accumulation in 15 *A. tenuissima* isolates (wort agar, 7 days, 25 °C) ranged from 0.8 to 710 μg/g [29]. In 17 strains isolated from grain in the Novosibirsk region (rice grain, 14 days, 25 °C), the variation was more significant, from 0.405 to 26900 μg/g [14]. The *A. tenuissima* isolates showed a group response to the type of nutrient medium. On MEA the predominant accumulation occurred in 12 isolates of those tested, V-8 agar was preferable for 5 isolates, and another 5 isolates produced comparable amounts of AOL on all three substrates. On average, over the entire set of isolates, the level of AOL accumulation decreased as MEA > V-8 > HAY. The toxin concentration of 10 μg/g or more was detected in

17 cultures on MEA, in 16 cultures on V-8, and in 10 cultures on HAY. Overactive production (above 100 μg/g) was noted on MEA and V-8, respectively, in 7 and 2 isolates, but it was not observed on HAY. In general, as followed from the mean values for the entire set, the greatest metabolic response in *A. tenuissima* was achieved on MEA, while on HAY and V-8 it was slightly lower.

1. Alternariol (AOL) **production by monoconidial isolates of *Alternaria tenuissima* from grain feeds on different agar media (**7 days, 25 °C) (*M*±SEM)

Isolate No., n = 23	AOL, μg/g substrate			
	PCA	MEA	HAY	V-8
204/1	0.04±0.01	105±20	26±3	38±4
215/1	0.50±0.10	22±4	16±3	23±2
221/1	0.07±0.00	253±35	37±7	12±1
222	0.80±0.20	138±18	20±4	72±12
225	2.90±0.40	73±15	37±6	12±2
228	–	30±7	4.8±0.6	0.3±0.0
233/1	0.04±0.00	10±2	23±6	80±14
234/2	–	33±3	7.3±1.1	117±23
236/1	0.02±0.00	26±6	59±8	19±3
241/1	0.20±0.00	200±43	53±8	37±7
242/1	0.90±0.20	39±7	1.5±0.3	3.9±0.2
255	0.04±0.00	5.5±0.7	0.3±0.0	12±2
337/1	0.04±0.00	0.1±0.0	0.1±0.0	0.2±0.0
342/1	0.03±0.00	58±8	87±15	120±25
357/1	0.06±0.01	21±4	4.7±0.9	6.1±0.9
359/1	2.30±0.40	100±18	1.7±0.2	19±2
368/1	50.00±8.00	2.0±0.1	0.2±0.0	0.2±0.0
372/1	1.10±0.20	31±5	0.3±0.0	2.7±0.5
381/1	0.20±0.00	23±6	9.2±1.4	12±2
384/1	0.05±0.00	5.8±0.9	5.9±0.9	3.6±0.7
392	0.10±0.00	402±43	69±13	25±6
395/1	0.10±0.00	3.7±0.6	0.1±0.0	13±2
397	0.20±0.00	106±18	0.5±0.1	24±4
n^+ (n^{10}/n^{100})	21 (0/0)	23 (17/7)	23 (10/0)	23 (16/2)
Range	0.02-2.90	0.1-402	0.08-87	0.2-120
Average, n^+	0.5	73	20	28

N o t e. n — the number of tested isolates; n^+ — the number of AOL producing isolates; n^{10} — the number of isolates with AOL production of > 10 μg/g; n^{100} — the number of isolates with AOL production of > 100 μg/g; a dash means that mycotoxin was not detected (detection limit of 0.01 μg/g); PCA — potato-carrot agar, MEA — malt extract agar, HAY — hay infusion agar, V-8 — vegetable juice agar.

Table 2 shows that the range of fluctuations in the AOL concertation on MEA and V-8 for *A. alternata* was 4 and 5 orders of magnitude. It was just as wide as in several *A. alternata* strains (rice grain, 14 days, 25 °C) isolated from grain in the Novosibirsk region [14]. The toxin was not detected in four isolates on PCA (Nos. 223/2, 380/1, 385/1 and 388/1), in three isolates on HAY (Nos. 216/2, 388/1 and 418/4) and in two on V-8 (Nos. 216/2 and 418/4). The average value for the set of isolates (1.3 μg/g, with 0.03 to 5.3 μg/g in range) on PCA was significantly lower than on other nutrient media.

2. Alternariol (AOL) **production by monoconidial isolates of *Alternaria alternata* from grain feeds on different agar media** (7 days, 25 °C) (*M*±SEM)

Isolate No., n = 20	AOL, μg/g substrate			
	PCA	MEA	HAY	V-8
210	0.3±0.0	155±24	100±20	267±38
216/2	2.2±0.3	2.8±0.4	–	–
219	0.6±0.1	3.1±0.5	2.8±0.5	2.8±0.4
220	0.1±0.0	125±25	23±4	89±10
223/2	–	272±38	1.9±0.3	3.5±0.5
227/1	3.3±0.7	133±30	10±2	19±3
233/2	0.4±0.1	114±20	25±4	13±2
235/2	0.1±0.0	130±30	70±12	70±10
238/1	0.3±0.0	6.5±1.2	27±6	10±2
342/2	0.03±0.01	3.3±0.6	8.9±1.3	2.0±0.2
358/1	0.5±0.1	20±3	11±2	38±3
377/1	0.9±0.2	10±2	14±3	14±3
380/1	–	0.2±0.0	0.3±0.1	0.4±0.1

Continued Table 2

383/1	0.1±0.0	43±7	35±7	50±9
385/1	–	246±39	71±15	365±43
388/1	–	0.04±0.01	–	0.07±0.01
390/1	0.6±0.2	80±15	57±10	461±70
396/1	2.2±0.4	72±10	48±8	19±3
399	3.6±0.8	7.0±1.3	53±8	40±8
418/4	5.3±1.2	0.1±0.0	–	–
n^+ (n^{10}/n^{100})	16 (0/0)	20 (12/7)	17 (13/0)	18 (12/3)
Range	0.03-5.3	0.04-272	0.3-100	0.07-267
Average, n^+	1.3	71	33	81

N o t e. n — the number of tested isolates; n^+ — the number of AOL producing isolates; n^{10} — the number of isolates with AOL production of > 10 μg/g; n^{100} — the number of isolates with AOL production of > 100 μg/g; a dash means that mycotoxin was not detected (detection limit of 0.01 μg/g); PCA — potato-carrot agar, MEA — malt extract agar, HAY — hay infusion agar, V-8 — vegetable juice agar.

The toxin production was comparable for V-8 and MEA (81 and 71 μg/g, respectively) and slightly less for HAY (33 μg/g). On PCA, producers with high activity were not detected at all, while on the other three media, the AOL accumulation of more than 10 μg/g occurred in 12-13 isolates. Ultra-high activity of 100 μg/g and more not observed on HAY, was established for 3 isolates on V-8 and for 7 isolates on MEA.

A. alternata and *A. tenuissima* could be grouped according to the response to the medium composition, with predominant accumulation on MEA (6 isolates), on HAY (2 isolates with amounts comparable to those on MEA) and V-8 (3 isolates), as well as on all three media (5 isolates). There were only few isolates producing the largest amounts of toxin on V-8 (No. 390/1) and with approximately equal AOL accumulation on PCA and MEA (No. 216/2). The isolate No. 418/4 from barley grain showed the abnormal response with the highest accumulation on PCA. In general, the highest metabolic response in *A. alternata* occurred on MEA and V-8 media. A more pronounced synthesis of AOL on these two media compared to wort agar and YES was previously shown by us for some strains identified as *A. tenuissima* and *A. alternata* [30].

Despite the obvious differences in response to the type of growth medium, substrate profiles of toxin production, and wide range of variation of AOL amounts in *A. tenuissima* and *A. alternata* (see Tables 1, 2), an estimate based on the total number of producers and the proportion of highly active and overactive isolates shows the highest level of realization of AOL biosynthetic potential of *A. tenuissima* and *A. alternata* on MEA. The total amount of AOL accumulated under these conditions for both species was practically the same, up to 73 and 71 μg/g, respectively.

A. arborescens isolates produced AOL in all media (Table 3), but in the amounts which varied significantly less than in the two species described above (see Tables 1, 2), being 1-2 orders of magnitude. However, the available set size was noticeably inferior to that for *A. tenuissima* and *A. alternata*; therefore, it is possible to state this fact, but a direct comparison is hardly correct. Indeed, for *A. arborescens*, significant fluctuations have been described; for example, for single strains from grain in the Novosibirsk region, the range of fluctuations in the amount of AOL was extremely wide [14]. However, in previous testing three strains of this species isolated from corn and sunflower seeds, the intensity of AOL production on MEA (3.3-36 μg/g) was quite comparable with fluctuations within the same order of magnitude [31].

Among the isolates, there were those with the highest AOL production on HAY and V-8, as well as on HAY and MEA. We would like to note that abnormal response to the type of medium is also possible, e.g., for the *A. arborescens* No. 100041 isolated in 2007 from wheat leaves in Syria the AOL level in the series PCA > MEA > HAY, V-8 decreased from 15±3 to 0.03±0.01 μg/g (unpublished

authors' data). The same type of metabolic response was characteristic of the *A. alternata* strain No. 418/4, which we isolated from barley grain (see Table 2). In the set as a whole, the highest AOL production occurred on V-8, HAY, and MEA media. The total AOL accumulation under these conditions was 106, 64, and 31 μg/g, respectively.

3. Alternariol (AOL) **production by monoconidial isolates of *Alternaria arborescens* from grain feeds on different agar media** (7 days, 25 °C) (M±SEM)

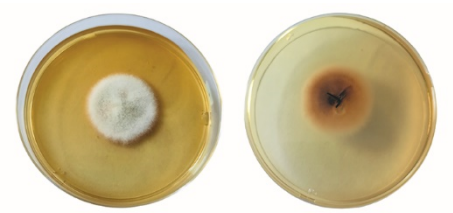
Isolate No., n = 8	AOL, μg/g substrate			
	PCA	MEA	HAY	V-8
3/3	1.9±0.3	79±14	117±22	173±30
15/6	1.5±0.3	10±2	11±3	20±6
19/4	0.8±0.1	4.4±0.5	47±11	8.8±1.6
39/4	0.9±0.1	4.0±0.5	6.8±1.2	13±4
338/1	1.5±0.2	77±13	133±20	12±2
157011	1.9±0.3	69±14	113±17	129±25
158011	1.3±0.2	2.0±0.2	69±13	488±38
529051	0.9±0.2	6.7±1.3	11±2	4.3±0.8
n^+ (n^{10}/n^{100})	8 (0/0)	8 (3/0)	8 (7/3)	8 (6/3)
Range	0.8-1.9	2.0-79	6.8-133	4.3-488
Average, n^+	1.3	31	64	106

N o t e. n — the number of tested isolates; n^+ — the number of AOL producing isolates; n^{10} — the number of isolates with AOL production of > 10 μg/g; n^{100} — the number of isolates with AOL production of > 100 μg/g; a dash means that mycotoxin was not detected (detection limit of 0.01 μg/g); PCA — potato-carrot agar, MEA — malt extract agar, HAY — hay infusion agar, V-8 — vegetable juice agar.

Differently directed shifts in the intensity of AOL biosynthesis on the growth media panel, which we observed in isolates of all three species, are possibly associated with intraspecific individual or group peculiarities of genome functional activity with the participation of regulators of metabolic processes. A more detailed study of this issue is of particular value for the development of the chemotaxonomy of *Alternaria* fungi, which has received increasing attention in recent years [32-34]. Our findings on the advantage of the commercial substrate MEA, as well as two other mycological media, V-8 and HAY are important to improve lab techniques for studying biosynthetic capabilities of these microscopic fungi, which was started in 1990th [35, 36] and is still discussed in the scientific papers [14, 37].

All fungal isolates which, on day 10 of growth at 25 °C on YES medium, formed a weakly colored aerial mycelium and colonies of different structures and densities, were assigned to the *A. infectoria* species group. For 13 of them, there was a distinct similarity in cultural and morphological characteristics (with small differences). The diameter of the colony was 45-60 mm, the structure of the aerial mycelium was predominantly cotton-like (from sparse to more dense), in its color a white-pink tone dominated which interspersed with gray, beige or olive-gray tones interspersed, the reverse side of the colony was light brown or dark brown, mainly with radial grooves. In the cultures possessing such characteristics AOL was not detected on the entire panel of growth media, and this result was consistent with that previously obtained on single isolates [25]. However, one of the cultures, *A. infectoria* No. 6/10 significantly differed from the others in growth rate (colony of 80 mm in diameter), low, relatively dense, slightly heavy aerial mycelium of light beige color with a pink sector (20 mm), the reverse side of the colony had a light brown color with radial grooves, with light orange in the sector area (Fig.). This isolate turned out to be an active producer of AOL with a sharp increase in activity on V-8 (220±30 μg/g) vs. 2.0±0.2; 14±3 and 18±4 μg/g on PCA, MEA and HAY, respectively. We also noted a similar response to a change in the growth medium in some members of *A. alternata* and *A. tenuissima* (see Tables 1, 2). According to German researchers, two isolates of *A. infectoria* from grain in the Novosibirsk region also produced AOL in contrasting amounts, the extremely low and ultrahigh [14]. Interestingly, recently, when studying a group of *A. infectoria* species on a

modified YES medium, two morphological types differing in pigmentation were revealed [38]; however, unfortunately, their ability to form toxins was not assessed.



Macroscopic characteristics of the *Alternaria* infectoria group isolate No. 6/10 (yeast extract sucrose agar, YES, 10 days, 25 °C): 1 — top view, 2 — back view.

The ability of *A. infectoria* to produce AOL is still open [1]. Most publications report that its synthesis is not characteristic of this group of species [18]; however, in several studies, toxinogenic producers were found among isolates [14, 16, 34]. According to the latest data, this group is characterized by production of toxins of another structural series, the perylenequinones [14, 16].

This work submits data on a cumulative assessment of *A. tenuissima*, *A. alternata* and *A. arborescens* from grain feeds based on a series of indicators, which experimentally confirms the pronounced and almost identical ability of the isolates to produce AOL. These results generally correspond to those obtained earlier for some representatives of these species [29-31], and also agree with the data of foreign researchers for species found in grain and grain products in Argentina, Brazil [7-9], Mediterranean countries, Slovakia, Italy [10, 11, 39] and Korea [13].

The principle of a set formation we used (1 isolate—1 sample) allows us for the first time to conclude about the high toxigenic potential of populations of three small-spore species, the *A. tenuissima*, *A. alternata*, and *A. arborescens* and their involvement in the AOL contamination of grain feed. According to recent data, AOL contamination in Russia in recent years has acquired particular relevance for corn feed grain [40] and grain-based mixed feeds [41].

Thus, our research revealed that among the *Alternaria* fungi colonizing grain fodder, populations of three morphological species, *A. tenuissima*, *A. alternata* and *A. arborescens* are capable of active alternariol production (more than 10 μg/g growth medium for most isolates) and may be involved in feed contamination, while the contribution of the *A. infectoria* group is unlikely. Further development of the population approach used in the presented work is important for improving methods of assessing the risks of feed contamination with toxicants of mycogenic origin. The successful use of typical mycological media for *in vitro* express testing of isolates confirms that it is possible to combine the control of toxigenic potential with species identification. The correspondence between morpho-cultural features and the phylogenetic position of these fungi, on the one hand, and the profile of their toxic secondary metabolites, on the other, remains a key component of the database, which has been actively constructed in recent years to clarify systematics of the genus *Alternaria*.

REFERENCES

1. Tralamazza S.M., Piacentini K.C., Iwase C.H.T., De Oliveira Rocha L. Toxigenic *Alternaria* species: impact in cereals worldwide. *Current Opinion in Food Science*, 2018, 23: 57-63 (doi: 10.1016/j.cofs.2018.05.002).
2. Ostry V. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal*, 2008, 1(2): 175-188 (doi: 10.3920/WMJ2008.x013).
3. EFSA on Contaminants in the Food Chain (CONTAM). Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal*, 2011, 9(10): 2407 (doi: 10.2903/j.efsa.2011.2407).
4. Lou J., Fu L., Peng Y., Zhou L. Metabolites from *Alternaria* fungi and their bioactivities. *Molecules*, 2013, 18(5): 5891-5935 (doi: 10.3390/molecules18055891).
5. Lee H.B., Patriarca A., Magan N. *Alternaria* in food: ecophysiology, mycotoxin production and

toxicology. *Mycobiology*, 2015, 43(2): 93-106 (doi: 10.5941/MYCO.2015.43.2.93).
6. Mao Z., Sun W., Fu L., Luo H., Lai D., Zhou L. Natural dibenzo-α-pyrones and their bioactivities. *Molecules*, 2014, 19(4): 5088-5108 (doi: 10.3390/molecules19045088).
7. Patriarca A., Azcarate M.P., Terminiello L., Fernández Pinto V. Mycotoxin production by *Alternaria* strains isolated from Argentinian wheat. *International Journal of Food Microbiology*, 2007, 119(3): 219-222 (doi: 10.1016/j.ijfoodmicro.2007.07.055).
8. Mašková Z., Tančinová D., Barboráková Z., Felšöciová S., Cisarová M. Comparison of occurrence and toxinogenity of *Alternaria* spp. isolated from samples of conventional and new crossbred wheat of Slovak origin. *Journal of Microbiology, Biotechnology and Food Sciences*, 2012, 1(4): 552-562 (doi: 10.15414 /jmbfs.2016.5.6.552-556).
9. Oviedo M.S., Sturm M.E., Reynoso M.M., Chulze S.N., Ramirez M.L. Toxigenic profile and AFLP variability of *Alternaria alternata* and *Alternaria infectoria* occurring on wheat. *Brazilian Journal of Microbiology*, 2013, 44(2): 447-455 (doi: 10.1590/S1517-83822013000200017).
10. Andersen B., Nielsen K.F., Fernández Pinto V., Patriarca A. Characterization of *Alternaria* strains from Argentinean blueberry, tomato, walnut and wheat. *International Journal of Food Microbiology*, 2015, 196: 1-10 (doi: 10.1016/j.ijfoodmicro.2014.11.029).
11. Ramires F.A., Masiello M., Somma S., Villani A., Susca A., Logrieco A.F., Luz C., Meca G., Moretti A. Phylogeny and mycotoxin characterization of *Alternaria* species isolated from wheat grown in Tuscany, Italy. *Toxins*, 2018, 10(11): 472 (doi: 10.3390/toxins10110472).
12. Li F., Toyazaki N., Yoshizawa T. Production of *Alternaria* mycotoxins by *Alternaria alternata* isolated from weather-damaged wheat. *Journal of Food Protection*, 2001, 64(4): 567-571 (doi: 10.4315/0362-028x-64.4.567).
13. Nguyen T.T.T., Kim J., Jeon S.J., Lee C.W., Magan N., Lee H.B. Mycotoxin production of *Alternaria* strains isolated from Korean barley grains determined by LC-MS/MS. *International Journal of Food Microbiology*, 2018, 268: 44-52 (doi: 10.1016/j.ijfoodmicro.2018.01.003).
14. Zwickel T., Kahr S.M., Rychlik M., Müller E.H. Chemotaxonomy of mycotoxigenic small-spored *Alternaria* fungi — do multitoxin mixtures act as an indicator for species differentiation? *Frontiers in Microbiology*, 2018, 9: 1368 (doi: 10.3389/fmicb.2018.01368).
15. Andersen B., Thrane U. Secondary metabolites produced by *Alternaria infectoria* and their use as chemotaxonomic markers. *Mycotoxin Research*, 1996, 12(2): 54-60 (doi: 10.1007/BF03192262).
16. Andersen B., Thrane U. Differentiation of *Alternaria infectoria* and *Alternaria alternata* based on morphology, metabolite profiles, and cultural characteristics. *Canadian Journal of Microbiology*, 1996, 42(7): 685-689 (doi: 10.1139/m96-093).
17. Andersen B., Krøger E., Roberts R.G. Chemical and morphological segregation of *Alternaria alternata*, *A. gaisen* and *A. longipes*. *Mycological Research*, 2001, 105(3): 291-299 (doi: 10.1017/S0953756201003446).
18. Pinto V.F., Patriarca A. *Alternaria* species and their associated mycotoxins. In: *Mycotoxigenic Fungi: Methods in Molecular Biology*, *vol. 1542*. A. Moretei, A. Susca (eds.). Humana Press, New York, NY, 2017: 13-32 (doi: 10.1007/978-1-4939-6707-0_2).
19. Lawrence D.P., Rotondo F., Gannibal P.B. Biodiversity and taxonomy of the pleomorphic genus *Alternaria*. *Mycological Progress*, 2016, 15(1): article 3 (doi: 10.1007/s11557-015-1144-x).
20. Gannibal Ph.B., Yli-Mattila T. *Mikologiya i fitopatologiya*, 2005, 39(4): 13-23 (in Russ.).
21. Frisvad J.C., Smedsgaard J., Larsen T.O., Samson R.A. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Studies in Mycology*, 2004, 49: 201-241.
22. Frisvad J.C., Hubka V., Ezekiel C.N., Hong S.-B., Nováková A., Chen A.J., Arzanlou M., Larsen T.O., Sklenár F., Mahakarnchanakul W., Samson R.A., Houbraken J. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Studies in Mycology*, 2019, 93: 1-63 (doi: 10.1016/j.simyco.2018.06.001).
23. Simmons E.G. *Alternaria. An identification manual*. CBS, Utrecht, 2007.
24. *Introduction to food- and airborne fungi*. R.A. Samson, E.S. Hoekstra, J.S. Frisvad, O. Filtenborg (eds.). CBS, Utrecht, 2000.
25. Burkin A.A., Kononenko G.P. *Immunopatologiya, allergologiya, infektologiya*, 2009, 2: 7-8 (in Russ.).
26. Gannibal F.B. *Mikologiya i fitopatologiya*, 2004, 38(3): 19-28 (in Russ.).
27. Gannibal F.B. *Mikologiya i fitopatologiya*, 2008, 42(4): 359-368 (in Russ.).
28. Gagkaeva T.Yu., Gannibal F.B., Gavrilova O.P. *Zashchita i karantin rastenii*, 2012, 1: 37-42 (in Russ.).
29. Piryazeva E.A., Kononenko G.P. *Materialy chetvertogo s"ezda mikologov Rossii «Sovremennaya mikologiya v Rossii», vol. 1* [Proc. Fourth Congress of Russian mycologists «Modern mycology in Russia»]. Moscow, 2017: 175-177 (in Russ.).
30. Ustyuzhanina M.I., Burkin A.A., Kononenko G.P., Piryazeva E.A., Zotova E.V. Alternative assay media for alternariol production by *Alternaria* species. *Proc. of the VIII International Conference on Environmental, Industrial and Applied Microbiology — BioMicroWorld2018 «Global progress in applied microbiology: a multidisciplinary approach»*. A. Méndez-Vilas (ed.). Formatex Research Center, Badajoz, Spain, 2018: 1-5.
31. Kononenko G.P., Ustyuzhanina M.I., Orina A.S. Multi-substrate screening the ability to produce

alternariol among *Alternaria arborescens* strains. *Journal of Veterinary Science & Technology*, 2019, 10: 41-42.
32. Andersen B., Krøger E., Roberts G. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research*, 2002, 106(2): 170-182 (doi: 10.1017/S0953756201005263).
33. Andersen B., Sørensen J.L., Nielsen K.F., van den Ende B.G., de Hoog S. A polyphasic approach to the taxonomy of the *Alternaria infectoria* species-group. *Fungal Genetics and Biology*, 2009, 46: 642-656 (doi: 10.1016/j.fgb.2009.05.005).
34. Patriarca A., da Cruz Cabral L., Pavicich M.A., Nielsen K.F., Andersen B. Secondary metabolite profiles of small-spored *Alternaria* support the new phylogenetic organization of the genus. *International Journal of Food Microbiology*, 2019, 291: 135-143 (doi: 10.1016/j.ijfoodmicro.2018.11.022).
35. Kostecki M., Grabarkiewicz-Szczęsna J., Chelkowski J. Biosynthesis and preparation of five *Alternaria* metabolites. *Mycotoxin Research*, 1991, 7(1): 3-7 (doi: 10.1007/BF03192156).
36. Häggblom P., Hiltunen M. Regulation of mycotoxin biosynthesis in *Alternaria*. *Mycotoxin Research*, 1991, 7(1): 8-10 (doi: 10.1007/BF03192157).
37. Oviedo M.S., Ramirez M.L., Barros G.G., Chulze S.N. Impact of water activity and temperature on growth and alternariol and alternariol monomethyl ether production of *Alternaria alternata* isolated from soybean. *Journal of Food Protection*, 2010, 73(2): 336-343 (doi: 10.4315/0362-028X-73.2.336).
38. Kosiak B., Torp M., Skjerve E., Andersen B. *Alternaria* and *Fusarium* in Norwegian grains of reduced quality — a matched pair sample study. *International Journal of Food Microbiology*, 2004, 93(1): 51-62 (doi: 10.1016/j.ijfoodmicro.2003.10.006).
39. Logrieco A., Bottalico A., Solfrizzo M., Mulé G. Incidence of *Alternaria* species in grains from Mediterranean countries and their ability to produce mycotoxins. *Mycologia*, 1990, 82(4): 501-505 (doi: 10.2307/3760022).
40. Kononenko G.P., Burkin A.A., Zotova E.V., Smirnov A.M. *Rossiiskaya sel'skokhozyaistvennaya nauka*, 2019, 3: 28-31 (doi: 10.31857/S2500-26272019328-31) (in Russ.).
41. Kononenko G.P., Burkin A.A., Zotova E.V. *Veterinariya segodnya*, 2020, 1(32): 60-65 (doi: 10.29326/2304-196X-2020-1-32-60-65) (in Russ.).