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SYMBIOTIC RELATIONSHIPS BETWEEN AGGRESSIVE *Fusarium* AND *Alternaria* FUNGI COLONIZING OAT GRAIN

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Abstract

The mycobiota of cereal grain consist of diverse fungal community among which *Fusarium* and *Alternaria* species are the most abundant. In biotic community, the competition and cooperative behavior may impact interspecies interactions. In this paper, we first report significant correlations between abundance of these fungi in oat grain. The symbiotic relationships that were found are of key importance because of mycotoxin production by *Alternaria* and *Fusarium* fungi. qPCR was used to measure *Fusarium* and *Alternaria* DNA in grain of 21 varieties and breeding lines of oats (*Avena sativa* and *A. byzantina*) from VIR World Collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources), and mycotoxin deoxynivalenol (DON) level was evaluated by ELISA test. Gere, Veler, KSI 731/01, KSI 432/08 genotypes appeared to be the most infected by both *F. culmorum* and *Alternaria* fungi. The highest amounts of DON (up to 1179 µg/kg) occurred in the grain of the breeding line KSI 432/08, as well as in Belinda and Konkur varieties. Stipler variety was the most resistant to all studied fungi and did not accumulate DON. Oat genotypes characterized by larger grains were more susceptible to fungal infections, whereas those with high husk proportion contained more DON. A high positive correlation between the amounts of DNA of *F. culmorum* and the *Fusarium* species able to produce trichothecene mycotoxins (Tri-*Fusarium*) was shown ($r = +0.67$, $p < 0.001$), as well as between *F. culmorum* DNA level and DON accumulation ($r = +0.57$, $p < 0.01$). When colonizing the same substrate, aggressive *Fusarium* species and relatively weak *Alternaria* pathogens are in symbiotic relationships. In this, we found the significant positive correlations between the amounts of DNA of *Alternaria* fungi and *F. culmorum* ($r = +0.66$, $p < 0.01$), and between DNA level of *Alternaria* and Tri-*Fusarium* fungi ($r = +0.86$, $p < 0.001$). In this study, DON produced by *F. culmorum* had no allelopathic effect on *Alternaria* fungi. At the same time, any interspecies interactions between *Alternaria* fungi and *F. poae*, the most abundant species detected in oat grain, were not found. Undoubtedly, the functions of fungal complexes colonizing plants depend on the parameters of the environment and the relationships evolutionarily developed within natural communities.

Keywords: *Avena* L., oat, grain, fungi, *Fusarium*, *Alternaria*, DNA, qPCR, deoxynivalenol, symbiotic interactions

N.I. Vavilov wrote that the immunity of a plant to a disease must be considered as the result of the interaction of many components associated with the habitat [1]. The mutual interaction of pathogens in the mycobiota community of grain undoubtedly influences their vital activity, which ultimately affects the relations with the host plant. Therefore, selection for disease resistance can be successfully carried out only upon considering all environmental factors.

Fungi of *Fusarium* Link genus cause fusariosis, a harmful disease of cereals leading to a decrease in yield and deterioration of its quality. The peculiarity of this disease lies in its specific etiology, i.e. a variety of *Fusarium* genus species

are involved in the infectious process. Thus, 10 to 20 species can coexist in one sample of grain with the explicit dominance of one species or group of species most adapted to the specific environmental conditions. *F. culmorum* (W.G. Smith) Sacc. is an aggressive pathogen of cereal crops in regions with a temperate climate, which can also infect many other plant species. The fungus is most frequently noted in Central and Northern Europe [2, 3], in Russia — in the Central, Central Black Earth and North-West regions [4-6]. *F. culmorum* produces deoxynivalenol (DON), 3-acetate-DON and less often nivalenol [7]. These mycotoxins belong to the most widespread and studied group of trichothecene metabolites, which are a significant threat when using contaminated grains. There are models for assessing the development of fusariosis and the accumulation of mycotoxins, taking into account the influence of the variety, environment, agronomic methods, but actual fungi and mycotoxins contamination of grain does not always corresponds to the forecasted one, which in the opinion of many researchers may be due to the effect of the concomitant mycobiota [8-10]. Indeed, usually fusarium fungi are not the only ones in the grain mycobiota and coexist with its other representatives.

In nature, all fungi inevitably interact directly or indirectly — both in a contact manner in competition for the substrate and allelopathically due to the influence of secondary metabolites accumulated in the substrate as well as volatile organic compounds, the functions of which are still not sufficiently deciphered [11-13]. Fungi of the *Alternaria* Nees genus presented in grain alongside *Fusarium* fungi are isolated at a high frequency [14-16]. About 30 species of the *Alternaria* genus are found in grain crops, the taxonomic position of many of which remains unclear [15, 17]. Small-spore species *A. alternata* (Fr.) Keissl, *A. tenuissima* (Kunze) Wiltshire and *A. infectoria* E.G. Simmons are most often mentioned; these species have similar morphological features [17-18], which often leads to their false identification. Meanwhile, according to some data, these species differ in ability to produce secondary metabolites, for example, alternariol (AOL) [18], and, as a consequence, to affect the rest of mycobiota. It is shown that the presence of *Alternaria* corresponds to various contamination of grain with other fungi. Thus, it was reported that the amount of *Alternaria* spp. DNA in wheat correlated with the content of *F. graminearum* Schwabe DNA negatively in awns and grain [19] and *A. alternata* infestation reduced the accumulation of DON in grain [20]. However, other researchers, despite notable amounts of *Fusarium* and *Alternaria* DNA, did not reveal a reliable relationship between the abundance of these fungi on the grain of barley, rye and wheat [21].

Oat (*Avena* L.) is a hulled crop, the grain of which is largely affected by fungi of varying degrees of pathogenicity [22]; interactions between them during colonization of plant tissue have been studied insufficiently. For grain damage, there are few reports on the mutual influence of typical members of *Fusarium* and *Alternaria* coexisting on the same substrate.

This paper is the first report on the symbiotic character of the relationship between *Fusarium* and *Alternaria* fungi living on the oat grain, which is of great practical importance, since these fungi produce mycotoxins that are dangerous to humans and animals.

Our aim was to study the interaction between typical members of the natural mycobiota of oat grain, *Alternaria* and *Fusarium* fungi, by assaying DNA of these pathogens in oat grain and mycotoxin contamination.

Techniques. The contamination of grains with *Fusarium* and *Alternaria* fungi was evaluated in breeding varieties and promising lines of *Avena sativa* L. (19 genotypes) and *A. byzantina* C. Koch (varieties Medved and CDC Dancer) of different ecogeographical origin, preserved in the VIR world collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources). The plants of each genotype

were grown in rows of 1 m length in two replicates (experimental station, Pushkin, Leningrad Region, 2015). During earing, plants were inoculated with a suspension of conidia and mycelia (1.3×10^7 CFU/ml, 50 ml/m²) of four *F. culmorum* strains, MFG 58219, 58562, 58578, 58585 (Collection of microorganisms, Laboratory of Mycology and Pathology, All-Russian Research Institution of Plant Protection). The oats variety Borrus previously characterized as medium-resistant to grain fusariosis [23] were additionally sown as a non-inoculated control. This area was treated with sterile water.

After harvesting and threshing, 1000 grain weight at a 12 % humidity was recorded and the proportion of the flower glume according to the guidelines [24]. For each genotype, 1000 grain weight was determined by weighing two 250-grain portions per sample and doubling the obtained value. The hull content was evaluated by weighing 4 portions of grain (50 pieces each), two with flower glumes retained, and two with flower glumes manually removed, calculating the percentage of hull weight from the total grain weight. If the discrepancy between the two measurements did not exceed 5 % of the average, this value was accepted as average weight. With greater differences, a new portion of grains was weighed and the average value between the nearest indicators was again calculated. The grain of each sample (10 g) was homogenized in sterile milling pots in a Tube Mill Control mill (IKA, Germany) at 25,000 rpm for 25 seconds. The milled meal was stored at -20 °C until extraction of DNA and mycotoxins.

DNA was isolated from 200 mg of grain meal by the adapted CTAB method [25]. DNA of *F. culmorum* and *Alternaria alternata* (Fr.) Keiss fungi strains was obtained from actively growing mycelium using a set of Genomic DNA Purification Kit reagents (Thermo Fisher Scientific, USA) in accordance with the attached protocol. The DNA concentration was evaluated in a Qubit 2.0 fluorimeter with a set of Quant-iT dsDNA HS Assay Kit reagents (Thermo Fisher Scientific, USA). Fungal DNA preparations were diluted to a concentration of 10 ng/ml and used to construct a calibration curve (10-fold serial dilutions from 10^{-1} to 10^{-6} ng/ml). DNA isolated from the grain was adjusted to working concentrations of 2-50 ng/ml.

The content of *F. culmorum* DNA in the meal of the grain samples was evaluated by real-time quantitative PCR (qPCR) with SYBR Green [26] stain. The reaction was performed in a volume of 20 µl containing 4 µl of 5× qPCRmix-HS SYBR master mix (Evrogen, Russia), 300 nM of each primer (Evrogen, Russia) and 2 µl of DNA solution. Tri-*Fusarium*, *Alternaria* and *F. poae* DNA concentrations were determined by the qPCR method with TaqMan fluorescent probes [27-29] in a reaction volume of 20 µl containing 10 µl of 2× TaqAB master mix (AlkorBio, Russia), 300 nM of each primer, 100 nM of fluorescent probe (Eurogen, Russia) and 2 µl of DNA solution, applying the recommended protocols with the authors' modifications. Amplification was performed on a CFX96 Real-Time System thermal cycler (Bio-Rad, USA), with primary data processing using Bio-Rad CFX Manager 1.6 software. The concentration of fungal DNA was expressed as a fraction of the total DNA isolated from the oat meal (ng/ng of total DNA).

DON in the milled grain was determined by competitive enzyme immunoassay (ELISA). Mycotoxin was extracted from 1 g of meal by adding 5 ml of acetonitrile:water (84:16) and allowed for 14-16 hours with continuous stirring (300 rpm) on a S-3M shaker (ELMI, Latvia). ELISA was performed using a test system with a sensitivity limit of 20 µg/kg (VNIIVSGE, Russia). Optical density was measured on a LEDETECT 96 (Biomed, Austria) photometer at $\lambda = 492$ nm.

All laboratory tests were performed in at least two replicates. The results were processed using statistical packages of Microsoft Excel 2010 (Microsoft Cor-

puration, USA), Statistica 10.0 (StatSoft, Inc., USA) and 4.PAST [30] software. The table shows the mean ($\bar{X}$) with a standard error ($\pm$ SE); the figure presents the mean ($\bar{X}$) with standard deviation ($\pm$ SD). The relationships between the studied indicators were evaluated by the correlation analysis using the Pearson criterion. The calculated coefficients were considered statistically significant at $p < 0.05$.

Results. We analyzed genotypes of oats, represented by hulled varieties and perspective lines. In these genotypes, the 1000 grain weight varied within the 29.8-46.7 g range, the proportion of flower glumes in the total biomass was 22.8-28.6 % (Table 1).

1. The main parameters of oats (*Avena* L.) varieties and breeding lines from the VIR world collection (N.I. Vavilov All-Russian Institute of Plant Genetic Resources) used in the study (experimental station, Pushkin, Leningrad Region, 2015)

VIR catalogue No.	Variety, line	Origin	Hull content, % ($\bar{X}\pm$ SE)	1000 grain weight, g ($\bar{X}\pm$ SE)
k-14648	Argamak	Russia, Kirov Region	24.5 $\pm$ 3.2	36.9 $\pm$ 5.6
k-11840	Borris	Germany	28.3 $\pm$ 3.7	34.9 $\pm$ 5.3
k-15068	Konkur	Russia, Ulyanovsk Region	25.7 $\pm$ 3.4	42.5 $\pm$ 6.9
k-10841	Binuandorodu	Russia, Sakhalin Region	25.6 $\pm$ 3.4	34.4 $\pm$ 5.0
k-14329	Kouzan Zairai	Japan	24.5 $\pm$ 3.2	42.0 $\pm$ 6.4
k-13911	Kambulinskii	Russia, Leningrad Region	25.0 $\pm$ 3.3	43.0 $\pm$ 6.6
k-14911	Belinda	Sweden	25.5 $\pm$ 3.3	42.0 $\pm$ 6.4
k-15297	Geszty	Hungary	22.8 $\pm$ 2.7	29.8 $\pm$ 4.5
k-15301	CDC Dancer	Canada	23.7 $\pm$ 3.1	41.7 $\pm$ 6.3
k-15506	Fux	Germany	24.6 $\pm$ 3.2	39.4 $\pm$ 6.0
k-15442	Zalp	Russia, Moscow Region	25.8 $\pm$ 3.0	41.5 $\pm$ 6.3
k-15496	Stipler	Russia, Ulyanovsk Region	26.4 $\pm$ 3.5	44.5 $\pm$ 6.8
k-15444	Sapsan	Russia, Kirov Region	24.3 $\pm$ 3.2	43.8 $\pm$ 6.6
k-15494	Medved	Russia, Kirov Region	27.2 $\pm$ 3.8	46.7 $\pm$ 7.1
k-15348	Hurdal	Norway	24.5 $\pm$ 3.2	42.8 $\pm$ 6.5
k-15353	Odal	Norway	23.3 $\pm$ 3.1	40.4 $\pm$ 6.1
k-15611	Bessin	Norway	25.3 $\pm$ 3.3	44.4 $\pm$ 6.2
k-15612	Veler	Norway	24.2 $\pm$ 3.2	41.2 $\pm$ 6.3
k-15347	Gere	Norway	24.0 $\pm$ 3.1	37.8 $\pm$ 5.7
k-15326	KSI 432/08	Russia, Ulyanovsk Region	28.6 $\pm$ 3.7	42.9 $\pm$ 6.5
k-15327	KSI 731/01	Russia, Ulyanovsk Region	25.0 $\pm$ 3.3	43.1 $\pm$ 6.5

2. Primers and probes used and qPCR conditions in the study

Object (reference)	Primers, probes	Nucleotide sequence 5'→3'	Amplification protocol
<i>F. culmorum</i> [27]	FculC561f FculC614r	CACCGTCATTGGTATGTTGTCACT CGGGAGCGTCTGATAGTCG	2 min at 50 °C, 10 s at 95 °C; 40 cycles: 15 s at 95 °C, 60 s at 62 °C
<i>F. poae</i> [28]	TMpoe,f TMpoe,r TMpoe,p	GCTGAGGGTAAGCCGTCCTT TCTGTCCCCCTACCAAGCT TET-ATTTCCCAACTTCGACTCTCCGAGGA-BHQ1	15 s at 95 °C; 40 cycles: 15 s at 95 °C, 1 min at 60 °C
Tri- <i>Fusarium</i> [29]	TMTRI,f TMTRI,r TMTRI,p	CAGCAGMTRCTCAAGGTAGACCC AACTGTAYACRACCATGCCAAC Cy5-AGCTTGGTGTGGGATCTGTCCTTACCG-BHQ2	3 min at 95 °C; 40 cycles: 15 s at 95 °C, 60 s at 60 °C
<i>Alternaria</i> [30]	DirITSAlt InvITSAlt AltTM	TGTCTTTTGCGTACTTCTTGTTTCCT CGACTTGTGCTGCGCTC FAM-AACACCAAGCAAAGCTTGAGGGTACAAAT-TAMRA	3 min at 95 °C; 40 cycles: 10 s at 95 °C, 60 s at 60 °C, 3 s at 72 °C

N o t e. Tri-*Fusarium* – all kinds of *Fusarium* fungi, capable of producing trichothecene mycotoxins.

The amplification protocols are summarized in Table 2.

Along with *F. culmorum* in the grain of oats, other *Fusarium* fungi were identified, including those capable to synthesize mycotoxins of the trichothecene group. Therefore, we additionally estimated the DNA amount of *F. poae* which occurs in the oat grain mycobiota with a high frequency [5, 31, 32]. Also, using the group-specific primers, the amount of DNA of all *Fusarium* species capable of producing trichothecene mycotoxins (Tri-*Fusarium*), as well as DNA of *Alternaria* genus fungi were additionally determined.

In Borrus (non-inoculated control), fungal mass per total DNA in grain estimated by qPCR was $(1.45\pm0.24)\times10^{-6}$ ng/ng for *F. culmorum*, $(1.73\pm0.02)\times10^{-5}$

ng/ng for Tri-*Fusarium* group, $(2.23 \pm 0.33) \times 10^{-5}$ ng/ng for *F. poae*, and $(6.24 \pm 0.31) \times 10^{-7}$ ng/ng for *Alternaria*. Under *F. culmorum* inoculation, the abundance of these species varied significantly. Grain level of *F. culmorum* DNA ranged from 7.39×10^{-8} to 3.07×10^{-5} ng/ng total DNA and averaged $(5.3 \pm 0.42) \times 10^{-6}$ ng/ng (Fig.). The total Tri-*Fusarium* DNA ranged from 1.5×10^{-5} to 9.1×10^{-5} and averaged $(5.3 \pm 0.28) \times 10^{-5}$ ng/ng, *F. poae* DNA rang was 1.05×10^{-5} – 3.06×10^{-4} with average value of $(7.45 \pm 0.83) \times 10^{-5}$ ng/ng total DNA. For *Alternaria* DNA, these indicators were 3.9×10^{-7} to 5.4×10^{-5} and $(1.07 \pm 0.6) \times 10^{-5}$ ng/ng. Apparently, the *Alternaria* species are well adapted and occupies its niche among mycobiota, regardless of the influence of environmental conditions and the genotype of the host plant. The samples Gere, Veler, KSI 731/01, KSI 432/08 were the most infected by *F. culmorum* and *Alternaria* fungi.

Fungal DNA in grain under oat (*Avena L.*) plant inoculation with a suspension of MFG 58219, 58562, 58578, 58585 *Fusarium culmorum* strains (qPCR, $\bar{X} \pm SD$, experimental station, Pushkin, Leningrad Region, 2015).

The qPCR method makes it possible to evaluate the DNA content of the target in grain, but has its own limitations. Thus, the primers for quantification of Tri-*Fusarium* and *F. culmorum* species are designed based on the nucleotide sequence of genes that are present in the genome in single copies, *Tri-5* and *EF1*, respectively [26, 27]. In contrast, primers for the quantitative detection of *F. poae* fungus and *Alternaria* species are developed based on multicopy sequences of IGS and ITS regions of ribosomal DNA [28, 33]. The primary differences between the initial quantities of the amplified fragments allow comparing infection by one or another target pathogen among the genotypes themselves, but do not allow comparing the

DNA level for different objects with each other in the same sample.

ELISA showed that 100 % of the analyzed oat genotypes after artificial inoculation with *F. culmorum* contained DON (from 25 to 1179 $\mu\text{g/kg}$, 198 ± 57 $\mu\text{g/kg}$ on average). The greatest quantities of mycotoxin were noted in the grain of the KSI 432/08 breeding line, as well as in the Belinda and Konkur varieties. The Stipler variety, most resistant to infection of all fungi, had 33 $\mu\text{g/kg}$ DON. DON was not found in the grain of non-inoculated control (Borris variety).

3. Interrelation of indicators (*r*) in oat (*Avena L.*) genotypes under inoculation with *Fusarium culmorum* (experimental station, Pushkin, Leningrad Region, 2015)

Indicator	DNA content in grain				DON ac- cumulation
Indicator	<i>F. culmorum</i>	<i>F. poae</i>	Tri- <i>Fusarium</i>	<i>Alternaria</i>	
DNA content in grain:					
<i>F. poae</i>	−0.34				
Tri- <i>Fusarium</i>	0.67**	−0.25			
<i>Alternaria</i>	0.66**	−0.27	0.86**		
DON accumulation	0.57*	0.19	0.21	0.18	
Hull fraction	0.37	0.08	−0.06	0.01	0.49*
1000 grain weight	0.34	−0.25	0.45*	0.39	0.16

Note. DON — deoxynivalenol.

*, ** Correlation coefficients are statistically significant at $p < 0.05$ and $p < 0.001$.

Correlation analysis revealed a high reliable relationship between DNA levels of *F. culmorum* and Tri-*Fusarium* group ($r = +0.67$, $p < 0.001$), which demonstrates the success of the performed inoculation (Table 3). For *F. cul-*

morum, the DON producer, there was also a strong relationship between the DNA amount and DON accumulation ($r = +0.57$, $p < 0.01$).

The 1000 grain weight value significantly correlated with the amount of *Tri-Fusarium* DNA ($r = +0.45$, $p < 0.05$). Genotypes with larger grain were relatively more strongly infected with fungi, which may be due to a greater content of nutrients allowing the fungus to gain biomass. At the same time, an increase in hull fraction led to a grain significant contamination with mycotoxin DON. The flower glume prevents penetration fungi into grain rich in nutrient substrates, and contributes (probably due to structure and chemical composition) to the intensive production of secondary metabolites by *Fusarium* fungi.

A reliable positive relationship was between the DNA content of the *Alternaria* and *F. culmorum* fungi ($r = +0.66$, $p < 0.01$) and between the *Alternaria* DNA level and the entire diversity of *Fusarium* species forming trichothecene mycotoxins ($r = +0.86$, $p < 0.001$). Apparently, during the colonization of the common substrate by aggressive species of *Fusarium* and relatively weak *Alternaria* pathogens, mutually beneficial conditions for the development of both arise, so that their relationships can be characterized as symbiotic. Even a significant DON level in grain did not adversely affect growth of *Alternaria* fungi. At the same time, we did not observe any relationship between *Alternaria* fungi and *F. poae* pathogen, a relatively weak and the most abundant member of *Fusarium* genus on the grain. Consequently, no competition arises between these fungi for the nutrient substrate during oat grain colonization and their relationship can be characterized as commensalism.

It is known that some mycotoxins produced by fungi can play an important role in the relationship between fungi and host plants [2, 10, 14]. In our study, we did not find the allelopathic effect of *F. culmorum* mycotoxins on *Alternaria* fungi, although some researchers noted various types of interaction between representatives of these genus. In particular, the slowing of *A. alternata* growth under the influence of *F. graminearum* has been described in laboratory experiments with co-cultivation [34]. In such a mixed culture, DON production by *F. graminearum* was lower, and the production of zearalenone (ZEN) was higher than in the pure culture of this pathogen. Another group of researchers reports that the rate of colonization of wheat grains by *A. tenuissima* fungus and the amount of secondary metabolites generated, for example AOL, increased significantly if the grain was pre-treated with DON or XEN mycotoxins [35]. Grain treatment with AOL had a negligible effect on *F. culmorum*, but led to an increase in *F. graminearum* growth. At the same time, the presence of *Fusarium* fungi on ears did not affect the amount of AOL.

Thus, we have identified a symbiotic relationship between *Alternaria* and *Fusarium* fungi, colonizing oat grain. Statistical analysis confirmed that there is a significant positive relationship between the abundance of these fungi in grain, which is especially important when combining *Alternaria* fungi and the highly aggressive *F. culmorum* species. During oat grain colonization, there is no competition for the nutrient substrate between *F. poae*, which is considered a relatively weak pathogen, and *Alternaria* fungi, hence their relationship can be characterized as commensalism. The interaction of fungi and plants is evolutionary conditioned, and the knowledge of its mechanisms in complex ecosystems is necessary for their control.

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