Naturally Occurring *Aspergillus* Species and their Mycotoxigenic Potential from Hungarian Sorghum (*Sorghum Bicolor* L. Moench) Kernels

Hazai szemes cirok (Sorghum bicolor L. Moench) szemtermésen megjelenő Aspergillus fajok és a mikotoxin-termeléssel összefüggésbe hozható génjeik vizsgálata

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Abstract: This study examined and identified the occurring *Aspergillus* spp. from sorghum (*Sorghum bicolor* L. Moench) kernels. We examined the internal infections of sorghum grains in PPA medium. During our experiments, we also used molecular methods to identify *Aspergillus* species and their genes, which promote aflatoxin production. This study identified two different *Aspergillus* species: *A. flavus* and *A. oryzae*. During identifying the genes, we detected that some of our A. flavus species could be aflatoxin-producing isolates.

Keywords*: aflatoxin; sorghum; aflatoxigenic genes; molecular identification; toxigenic fungi*

Összefoglalás: A szemes cirok (Sorghum bicolor L. Moench) termesztése az utóbbi két év aszályos és kiszámíthatatlan csapadékeloszlású termesztési évei miatt a kukorica alternatívájaként hazánkban is egyre jobban a termesztők érdeklődésének a középpontjába került. A cirok növényvédelméről azonban kevés az elérhető információ, a szemtermésen előforduló egészséget károsító penészgombákról pedig még korlátozottabbak az ismeretek, ami azért problémás, mert a szemtermés takarmány alapanyagként és élelmiszerként egyaránt hasznosul. A gabonanövények szemterméseinek mikotoxinokkal való szennyezettsége az utóbbi termesztési év tapasztalatai alapján nagy problémát okoz a gyakorlatban; az előforduló gombák nagy része polifág, ráadásul szaprofitaként is előfordul a talajban, rezisztenciával pedig nem rendelkeznek a köztermesztésben lévő fajták. A mikotoxin termelő penészgombák közül az Aspergillus fajok szubtrópusi eredetű kórokozók, azonban a fent említett hazai időjárásbeli változások közül is elsősorban az emelkedő hőségnapok számának növekedése ezen kórokozók előfordulásának is kedvez. Mivel magyarországi termőhelyről származó információkban a szakirodalom sem bővelkedik, ezért választottuk kísérletünk alapjának a hazai területekről származó szemes cirok mintákat. Kísérleteink során 30 szemes cirok minta Aspergillus fajok által okozott belső fertőzöttségét vizsgáltuk laboratóriumi körülmények között. Vizsgálataink során a belső fertőzöttségi vizsgálatokhoz PPA (Pentachloronitrobenzene Peptone Agar) táptalajt alkalmaztunk. A tiszta tenyészetek létrehozása után az izolált Aspergillus fajokat molekuláris genetikai úton azonosítottuk a kalmodulin (CaM) génre specifikus primer párral (cmd5/cmd6; CL1/CL2A). A kísérleteink során a Gallo és mtsai. (2012) és Degola és mtsai. (2007) által leírt aflatoxin termelésben szerepet játszó géneket azonosítottuk, amely alapján

megállapítottuk, hogy az általunk azonosított A. flavus izolátumok némelyike lehetséges aflatoxin termelő.

***Kulcsszavak**: aflatoxin; szemes cirok; aflatoxin termeléssel összefüggésbe hozható gének; molekuláris genetikai vizsgálatok; mikotoxin-termelő gombák*

1. Introduction

Sorghum (Sorghum bicolor L. Moench) is the main staple crop in developing countries, used for both animal and human consumption; in the ranking, it is the fifth most harvested crop worldwide (FAOSTAT 2023). Due to its resistance to drought, efficient use of water, and heat tolerance, sorghum will become an essential plant for human nutrition in the near future; many studies in the literature mention it as the grain of the future (Chhikara et al. 2019; Meena et al. 2022; Safian et al. 2022; Bakari et al., 2023). In addition, regular consumption of sorghum-based products has many health benefits due to its strong antioxidant effect. It can lower cholesterol, have anti-inflammatory and anti-cancer properties, and reduce obesity, type II diabetes, and cardiovascular disease (Xiong et al. 2019). The genus Aspergillus includes a diverse group of species based on morphological, physiological, and phylogenetic traits that have significant implications for biotechnology, food production, indoor environments, and human health. Among them, fungal species belonging to the Aspergillus Flavi section are important from the point of view of agriculture, biotechnology, the food industry, and human and animal health (Frisvad et al. 2018).

Degola et al. (2007) used five primer pairs to specifically amplify the AflD, AflR, AflS, AflO, and AflQ genes associated with aflatoxin production in *Aspergillus* species. The obtained results supported the generally accepted paradigm, according to which the expression of the AflR gene is necessary for the large-scale production of the toxin. The regulatory role of the AflR and AflS genes in regulating the expression of structural genes has already been proven. As expected, the observed structural gene expression follows AflR and AflS transcription. Although AflD, AflO, and AflQ encode enzymes of the aflatoxin biosynthesis pathway, they did not experience the advantages of their use in the laboratory compared to other genes of the aflatoxin cluster. Gallo et al. (2012) also identified the two regulatory genes, AflR and AflS, and the structural genes, AflD, AflM, AflO, AflP, and AflQ. Primer pairs targeting these genes were used in the study of genes related to aflatoxin production. The correlations between the presence of the mentioned genes and the aflatoxin production of the groups were then observed in different groups. They found that only those strains that lacked the presence of three or more of the observed genes were unable to produce aflatoxin. In contrast, all of the aflatoxin-producing isolates showed the complete gene set. Thus, the lack of amplification of aflatoxin biosynthetic genes was consistently associated with the non-aflatoxigenic nature of the strains. Also, in their case, AflO, AflP, and AflQ were amplified genes in a large number of non-aflatoxin isolates.

2. Materials and Methods

2.1. Materials

The *Aspergillus* species were isolated from sorghum kernels, which were collected in several parts of Hungary. During the molecular identification, we identified 21 isolates and two different species. The isolates were: INVT_A001; INVT_A002, INVT_A003, INVT_A004, INVT_A005; INVT_A006, INVT_A007, INVT_A008, INVT_A009, INVT_A010,

INVT_A011, INVT_A012, INVT_A013, INVT_A014, INVT_A015, INVT_A016, INVT_A017, INVT_A018, INVT_A019, INVT_A020, INVT_A021, INVT_A022, INVT_A023. For the molecular identification of the promoting genes of aflatoxin production, these isolates have been used.

2.2. Molecular identifications

For the molecular genetic studies, we isolated the *Aspergillus* cultures into 90-mm-diameter petri dishes on PDA medium. For DNA extraction, we used the ZR Fungal/Bacterial DNA MiniPrep kit, following the manufacturer's instructions. DNA has been taken from fresh fungal mycelia, taking care not to contaminate the process. During the molecular genetic identifications, in the case of belonging to the genera *Aspergillus,* standard polymerase chain reaction (PCR) was used to amplify the CaM (calmodulin) gene region, CL1 (forward primer; 5'-GA(GA)T(AT)CAAGGAGGCCTTCTC-3') and CL2A (reverse primer; 5'-TTTTTGCATCATGAGTTGGAC-3') (O'Donnell et al. 2000). The Pro FlexTM thermocycler (Applied BiosystemsTM Singapore) was used for amplification with the conditions described by O'Donnell et al. 2000. The CaM PCR product was used as a template for DNA sequencing by Macrogen Europe (Macrogen Europe BV, Amsterdam). Sequences were compared using the US National Center for Biotechnology Information (NCBI) database, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. In addition, the sequences were also uploaded and validated in the NCBI Genebank database. During the PCR tests, we examined the presence of seven genes associated with mycotoxin production using the standard PCR method described by Degola et al. (2007) and Gallo et al. (2012). These primers were specific primers for AflR (forward: 5'-AAGCTCCGGGATAGCTGTA-3'). reverse: 5'-AGGCCACTAAACCCGAGTA-3'), AflS (forward: 5'-TGAATCCGTACCCTTTGAGG-3'; reverse: 5'-GGAATGGGATGGAGATGAGA-3'), AflD (forward: 5'-CACTTAGCCATCACGGTCA-3'; reverse: 5'- GAGTTGAGATCCATCCGTG-3'), AflM (forward: 5'-AAGTTAATGGCGGAGACG-3'; reverse: 5'- TCTACCTGCTCATCGGTGA-3'), AflP (forward: 5'-AGCCCCGAAGACCATAAAC-3'; reverse: 5'-CCGAATGTCATGCTCCATC-3'), AflO (forward: 5'- TCGTCCTTCCATCCTCTTG-3'; reverse: 5'-ATGTGAGTAGCATCGGCATTC-3'), AflQ (forward: 5'- TCGTCCTTCCATCCTCTTG-3'; reverse: 5'-ATGTGAGTAGCATCGGCATTC-3') genes, which genes play a role in the aflatoxin production of the fungus.

3. Results

3.1. Molecular identification of *Aspergillus* species

During the species-level identification of the members of the isolated *Aspergillus* genus, 23 pure cultures were created. After extracting the DNA of the 23 pure cultures, the PCR run was performed with the primer pair CL1/CL2A. The PCR products were prepared with the CL1/CL2A primer pair for the amplification of the DNAs. During the molecular genetic tests, 2 *A. oryzae* and 19 *A. flavus* species were identified.

3.2. Molecular identification of aflatoxigenic genes of the identified *Aspergillus* species

Gel electrophoresis was performed on the DNA of the 21 identified *Aspergillus* species with primer pairs of genes linked to aflatoxin production (AflD, AflM, AflO, AflP, AflQ, AflR, and AflS). During the experiment, the last two samples in the gel containing PCR products were the

positive and negative controls. In the case of the positive control, we used DNA samples from the collection of MATE, NVI, and the Department of Integrated Plant Protection. In the case of the negative control, the PCR product did not contain any DNA; molecular water was added to the PCR products instead. Table 1 contains the results obtained during gel electrophoresis for each gene-specific primer.

Table 1. Amplified target sequences of gene specific primers. In the table, positive results where the primer amplified the target sequence were marked with "+" and green, and "-" where the primer did not amplify the target sequence

N°	Code of isolate and DNA	Species	AflD	AflM	AflO	AflP	AflQ	AflR	AflS
1	INVT_A023	*Aspergillus flavus*	-	-	+	+	+	-	-
2	INVT_A022	*Aspergillus flavus*	-	-	-	-	-	-	-
3	INVT_A021	*Aspergillus flavus*	-	-	-	-	-	-	-
4	INVT_A020	*Aspergillus flavus*	+	+	+	+	-	+	+
5	INVT_A019	*Aspergillus flavus*	+	+	+	+	-	+	+
6	INVT_A018	*Aspergillus flavus*	-	-	+	-	-	-	-
7	INVT_A017	*Aspergillus flavus*	-	-	+	+	+	-	-
8	INVT_A016	*Aspergillus flavus*	-	-	+	-	-	-	-
9	INVT_A015	*Aspergillus flavus*	+	+	+	+	+	+	+
10	INVT_A014	*Aspergillus flavus*	+	+	+	+	+	+	-
11	INVT_A013	*Aspergillus flavus*	+	+	+	+	+	+	+
12	INVT_A012	*Aspergillus flavus*	+	+	+	-	+	+	+
13	INVT_A011	*Aspergillus flavus*	+	+	+	-	-	+	+
14	INVT_A010	*Aspergillus oryzae*	-	+	+	+	+	+	+
15	INVT_A009	*Aspergillus flavus*	+	+	+	+	-	+	+
16	INVT_A008	*Aspergillus oryzae*	+	+	-	-	-	+	+
17	INVT_A007	*Aspergillus flavus*	-	+	+	+	-	+	+
18	INVT_A006	*Aspergillus flavus*	+	+	+	-	-	+	+
19	INVT_A005	*Aspergillus flavus*	-	+	+	-	-	+	-
20	INVT_A004	*Aspergillus flavus*	+	+	+	-	-	+	+
21	INVT_A003	*Aspergillus flavus*	+	+	+	+	+	+	+
22	INVT_A002	*Aspergillus flavus*	+	+	+	+	+	+	+
23	INVT_A001	*Aspergillus flavus*	-	+	+	+	+	+	-

For samples INVT_A015 and INVT_A013, all primers were able to amplify the target sequence, which means that we got a positive result for all genes that help mycotoxin production. In the case of the INVT_A022 sample, we did not find the presence of a single gene. Some DNA from *Aspergillus flavus* and *A. oryzae* were put through gel electrophoresis.

The AflD gene was found at 852 base pairs on 12 of the pieces. The presence of the AflM gene was found at 470 base pairs in 15 *A. flavus* and 2 *A. oryzae* DNAs. The presence of the AflO gene was detected in 19 *A. flavus* and 1 *A. oryzae* cases. During gel electrophoresis, the bands appeared at 790 base pairs. In the case of the AflP gene, we found its presence at 870 bp in 12 *A. flavus* and 1 *A. oryzae*. The presence of the gene AflQ, which can be associated with aflatoxin production, was found in the DNA of 9 *A. flavus* and 1 *A. oryzae* of the isolated fungi. The presence of the AflR gene was detected during gel electrophoresis at 1079 bp, which occurred in 15 A. flavus and 2 A. oryzae samples. The presence of the AflS gene was detectable in 12 *A. flavus* and 2 *A. oryzae* cases at 684 base pairs.

4. Discussion

During the isolation of *Aspergillus* species, 23 pure cultures were created, from which 19 *A. flavus* and 2 *A. oryzae* were identified as molecular tools. All of the identified species belong to the section *Flavi*, which is extremely important from the point of view of agriculture, biotechnology, and human and animal health (Frisvad et al. 2018). *A. flavus* is a frequently occurring species of agricultural plant that is often isolated from many parts of the world, and due to their aflatoxin-producing ability, they pose a major threat during food and feed (Palencia et al. 2010; Riba et al. 2010). *A. oryzae* species have also been isolated in many cases, and due to their non-aflatoxin properties, the species is considered safe. Furthermore, the fermentation industry still uses the species for various fermentation processes (Chang and Ehrlich 2010). Species have also been identified in many cases under domestic conditions (Baranyi et al., 2015; Tóth et al., 2012; Dobolyi et al., 2013; Sebők et al., 2016).

In the experiments conducted by Degola et al. (2007) and Gallo et al. (2012), the association of different genes with aflatoxin production was investigated. In our experiments, based on their research, we searched for the presence of the most important genes from the point of view of aflatoxin production. In a 2012 experiment, Gallo and his colleagues discovered that non-aflatoxin isolates were capable of amplifying the AflQ gene in large numbers. The AflQ gene was amplified 10 times in the samples we examined (INVT_A023, INVT_A017, INVT_A015, INVT_A014, INVT_A013, INVT_A012, INVT_A010, INVT_A008, INVT_A002, INVT_A001), which suggests that the 10 species of *Aspergillus* are probably Gallo and others. In his 2012 research, it belongs to the group considered non-aflatoxin. However, since the presence or absence of a single gene does not clearly determine the mycotoxin production capacity, it is also important to evaluate the results of the other tested genes.

In the case of the AflR and AflS genes, the close correlation between the presence of the genes and their ability to produce aflatoxin has been demonstrated (Degola et al., 2007; Gallo et al., 2012). Thus, in the case of our own samples, the presence of these genes would make the production of aflatoxin probable. During our experiments, 14 samples (INVT_A020, INVT_A019, INVT_A015, INVT_A013, INVT_A012, INVT_A010, INVT_A011, INVT_A009, INVT_A008, INVT_A007, INVT_A006, INVT_A004, INVT_A003, INVT_A002) amplified the AflS gene. The AflR gene was detected in 17 samples (INVT_A001, INVT_A002, INVT_A003, INVT_A004, INVT_A005, INVT_A006, INVT_A007, INVT_A008, INVT_A009, INVT_A010, INVT_A011, INVT_A012, INVT_A013, INVT_A 014, INVT_A015, INVT_A019, INVT_A020). Degola et al.'s 2007 experiment indicated aflatoxin production capacity with high certainty in the presence of these genes. Therefore, in the case of the samples, it is likely that an *Aspergillus* species is capable of producing aflatoxin. In addition to all of this, it is important to highlight that the identification of the mentioned genes in the individual samples does not mean a clear aflatoxin production

capacity; it merely makes this capacity probable, since Gallo et al. (2012) also detected aflatoxin production only in the case of the combined presence of the tested genes during their 2012 experiment. During our experiments, we experienced the co-presence of the genes in the cases of samples INVT_A002, INVT_A003, INVT_A010, INVT_A013, INVT_A014, and INVT_A015.

5. Conclusions

Our data are first insights in the identification of *Aspergillus* species in sorghum grains in Hungary. Our results highlighted the appearance of *Aspergillus* species in sorghum grains and their capability to inhibit aflatoxin production. Reliable identification of *Aspergillus* species and defining their capacity for mycotoxin production are important for plant disease management and for food and feed safety. Our data can help in the mycotoxin regulation of cereals grown in small areas. Moreover, it could help sorghum breeders by handing over advanced knowledge on the variability of *Aspergillus* species.

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