Thesis of University Doctoral (Ph.D.) dissertation

INVESTIGATIONS ON AFLATOXIN PRODUCTION ON AGRICULTURAL COMMODITIES

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1. BACKGROUND AND OBJECTIVES OF THE DISSERTATION

Mycotoxins were discovered in the case of a mass turkey mortality caused by aflatoxin-containing feed in the 1960s. In 1974, about 100 people died by aflatoxicosis, while, in 2004, death cases of 125 people were caused by acute aflatoxicosis because of aflatoxin-containing food in Kenya (Probst et al., 2007). The International Agency for Research on Cancer recognized aflatoxin B1 as a part of the Group 1 risk category as the molecule was recognized to be carcinogenic to humans (IARC, 2012). For optimum production, the primary producer *A. flavus* filamentous fungus requires a warm temperature around 36-38ºC and a minimum humidity of 85% (Diener et al., 1987). Therefore, until now, the toxin contamination appeared mainly in warmer regions with a highly humid climate, *e.g*., in Africa and India. Unfortunately, the molds and the toxin had been detected in many more countries, including Hungary, because of climate change. In 2007 and 2012, Hungarian researchers detected high levels of aflatoxin B1 in Hungarian cereals, while *A. flavus* caused infection reached a higher level in 2017 (Szabó et al., 2018). According to Battilani et al. (2016), if the average temperature increases with 2 ºC, aflatoxin B1 contamination would increase significantly in areas in France, the Carpathian Basin, and the Balkans. While, if the average temperature increases with 5 ºC, all the Europe except the Scandinavian parts would be highly contaminated with aflatoxins. It also expresses that aflatoxin contamination and the control of that is a current and serious problem.

Nevertheless, approximately 60 % of the naturally occurring *A. flavus* strains can not produce aflatoxin B1 (Horn et al., 1999). These non-aflatoxigenic strains, due to their competition with the aflatoxigenic *A. flavus* fungi, can be effectively used pre- and postharvest to reduce the aflatoxin B1 contamination of corn, cotton or peanut commodities. As a biocontrol agent, non-aflatoxigenic *A. flavus* has been marketed as Afla-Guard (for corn and peanuts) and A. flavus AF36 (for pistachios, corn, and cottonseeds) in the USA to reduce aflatoxin contamination in fields (Brown et al., 1991; Abbas et al., 2011). The components of these products are endemic *A. flavus* strains from the same area where they are applied (Mauro et al., 2015).

The research aimed to isolate an aflatoxin non-producing *A. flavus* endemic in our area in Hungary, which has a high resistance to various stress effects and does not produce other secondary metabolites and could reduce the aflatoxin contamination in maize. Loss of the toxin-production ability in fungi is probably due to mutations (Adhikari et al., 2016), and it is essential to exclude other toxin-producing ability before using the non-aflatoxigenic *A. flavus* strain in biocontrol, as it may also increase health risk.

The scopes of the research were:

- To collect aflatoxin producing and non-producing *A. flavus* isolates from the feed, agricultural commodities, and raw materials and to identify these isolates by PCR;
- To examine the stress-tolerance, growth and aflatoxin production capacity of a reference strain and the *A. flavus* isolates on various media;
- To examine the growth of the fungal isolates and the reference strain on different corn hybrids;
- To analyze the fungal isolates capacity of secondary metabolite production.

2. MATERIALS AND METHODS

2.1 Isolation of fungal strains

From feed, agricultural commodities and raw materials, 72 *Aspergillus sp.* isolates were collected in 2013-2014 at the Central Laboratory of Agricultural and Food Products, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen. *A. flavus* NRRL 11611, NCAIM F 00952 was purchased from the National Collection of Agricultural and Industrial Microorganisms, Szent István University.

2.2 Nucleic acid-based identification

Fungal cells were disrupted by using MagNA Lyser Green (Roche) beads. The DNA was isolated by the Nucleospin Plant II kit (Macherey-Nagel) according to the manufactural description of the Genomic DNA from Plant. Three genes responsible for aflatoxin production of *A. flavus* were identified by multiplex PCR. In the PCR, three primer pairs of the following sequence were applied (Varga et al., 2011):

nor1: 5-ACC GCT ACG CCGGCA CTC TCG GCA-3

nor2: 5-GTT GGC CGC CAG CTT CGA CAC AGC-3

aflR-R: 5-TGG KGC CGA CTC GAG GAA YGG GT-3

aflR-F: 5-GGG ATA GCT GTA CGA GTT GTG CCA-3

omt1: 5-GTG GAC GGA CCT AGT CCG ACA TCA C-3

omt2: 5-GTC GGC GCC ACG CAC TGG GTT GGG G-3

Phusion High-Fidelity Kit (Thermo Scientific) was applied for the PCR reactions.

DNA fragments of ITS1-ITS4 region and cmd5-cmd6 (calmodulin) gene were also amplified by PCR for the genus and species identification of isolates (White et al., 1990; Hong et al., 2006).

Primers used:

ITS1: 5-TCC GTA GGT GAA CCT GCG G-3

ITS4: 5-TCC TCC GCT TAT TGA TAT GC-3

cmd5: 5-GTC TCC GAG TAC AAG GAG GC-3

cmd6: 5-TCG CCG ATA GAG GTC ATA ACG TG-3

Here, Phusion High-Fidelity Kit (Thermo Scientific) was used for the PCR reaction, too. After purification of the PCR product of ITS1- ITS4 primers using of NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) kit, it was sequenced by the Biomi Ltd. (Gödöllő, Hungary). The obtained data were analyzed using MEGA 7 program, and similarity search was done using the database of GenBank (Benson et al., 2013).

2.3 Examination of different culture conditions and stress conditions

The growth of *A. flavus* NRRL 11611 reference strain and its production of aflatoxin B1 were examined on various media:

- cornmeal agar media with different crude fat content
- malate agar medium
- cornmeal agar + inorganic salts medium
- cornmeal agar + glucose medium
- Czapek-Dox agar medium

The results were analyzed using t-probe Microsoft Excel software (at a significance level of $P<0.05$). The correlations between the crude fat content, flavonoid and polyphenol content and the measured aflatoxin B1 concentrations were analyzed by correlation analysis using Microsoft Excel software.

The morphological characteristics and the production of aflatoxin B1 of the reference strain were investigated on malate agar medium and different quantity of spores applied for the inoculation.

During stress examination, growth, sclerotium development, production of conidiospores, and pigmentation of the fungal isolates and *A. flavus* NRRL 11611 reference strain were monitored on malate agar and corn meal agar media. For stress, NaCl (sodium chloride) at concentrations of 1, 1.5, 2 and 2.5 M or SDS (sodium dodecyl sulfate) at concentrations of 0.05, 0.1, 0.5 and 0.75 g/l were applied in the media.

In each case, the fungi were cultivated for 5-7 days and at 30ºC in the dark.

Analysis of the medium composition:

- determination of the crude fat content: according to section 8.3 in MSZ EN ISO 11085: 2015

- determination of total phenolic content: by Folin-Ciocalteu method as gallic acid equivalent, according to Kaur and Kapoor (2002)
- determination of the total flavonoid content: according to Chang et al. (2002) as catechin equivalent
- determination of the D-glucose content: by the D-glucose Assay Kit (Megazyme, GOPOD-Format)
- determination of the nitrogen content: according to the MSZ EN ISO 5983-2:2009 standard

2.4 *In vitro* inoculation of corn kernels with *Aspergillus flavus* for aflatoxin B1 and other secondary metabolite analysis

50 g corn kernel was disinfected with 70% ethanol and washed thoroughly with distilled water and placed in a sterile Erlenmeyer flask before inoculation with 500 µl suspension of 10^6 fungal spores. It was incubated at 30 ºC for 7 days in the dark.

2.5 Measurement of secondary metabolites by HPLC

2.5.1 Sample preparation from corn kernels for HPLC

25 g of the corn kernels were weighted, and 2.5 g NaCl and 50 ml 80% methanol was added to it. It was homogenized thoroughly with a blender (Commercial Blender, Waring) at high speed for three minutes. It was then filtered through an MN 619 filter paper. I removed 10 ml of the filtered sample and diluted it with 40 ml of distilled water. After stirring it with a magnetic stirrer, I transferred 10 ml of this sample prep through an Afla-B™ immunoaffinity column (Romer) with a speed of 1-2 drops/min. The column was washed with 10 ml distilled water, and the elution of the bound aflatoxins was done with 5 ml of HPLC grade methanol (Sigma-Aldrich). The eluent was evaporated from the sample in a rotadest (Rotavapor R114; Buchi).

2.5.2 Sample preparation for HPLC-MS and HPLC

The agar medium, together with the one-week-old surface culture, was collected in sterile Stomacher homogenizer bag and, with 10 ml chloroform, it was homogenized in Stomacher homogenizer (Masticator, IUL Instruments) for 2 min, which process was repeated. The homogenized culture was filtered through filter paper (MN 619; Macherey-Nagel) into round

flasks and was evaporated in Rotavapor R114 (Büchi). After the addition of 2 ml mobile phase (methanol: water, 45:55), the solute was filtered through a Millex-GV 0.22 µm filter (Merck-Millipore). For the HPLC-MS technique, the inoculated and incubated corn kernels (as described in Section 2.4) were treated in the same way after grinding (Fente et al., 2001).

2.5.3 Measurement by HPLC

Sampler: Merck Hitachi AS-4000 Intelligent Autosampler

Pump: Merck Hitachi L-6200A Intelligent pump (flow rate: 1ml/min)

Detector: Fluorescence ex360nm, em440nm

Column: Genesis C18, 4.6x150 mm (4µm)

Loop volume: 20 µl

Mobile phase: methanol: water (45:55), isocratic

2.5.4 Measurement by HPLC-MS

Measurements were done by Dr. Lajos Nagy (Department of Applied Chemistry, Institute of Chemistry, Faculty of Science and Technology, University of Debrecen).

The HPLC-MS system contained a Waters Separations module (2695), a Waters 2996 Photodiode Array detector. Metabolites were separated using a Zorbax SB-C18, 4.6x75 mm, 3.5 µm column. The flow rate was 0.5 ml/min, the elution time 60 min, and the temperature 40ºC. The UV detection was performed at 225 nm. The gradient of methanol: H_2O was applied. Mass spectra were acquired using Bruker microTOF-Q mass spectrometer equipped with Atmospheric Pressure Photoionization (APPI) ion source. The temperature of the ion source was 390ºC. The data were analyzed using the Bruker Daltonics DataAnalysis software.

Analyzing the HPLC-MS results for the identification of several secondary metabolites, I applied the data from the publication of Uka et al. (2017).

2.6 Competition test

50 g of corn kernels of MV251 hybrid was weighed in a 500 ml sterile Erlenmeyer flask. I washed the corn seeds with 70% HYPO, then with sterile distilled water, and 70% ethanol solution, and again with sterile distilled water. The corn kernels were inoculated with 1 ml 10^6 fungal spores in different ratio of toxinogenic and non-aflatoxigenic *A. flavus* isolates: 100% toxinogenic; 100% non-aflatoxigenic; 50%:50% toxinogenic: non-aflatoxigenic; 30%:70%

toxinogenic: non-aflatoxigenic; 70%:30% toxinogenic: non-aflatoxigenic isolates. The kernels were incubated at 30 ºC, for seven days in the dark. After the incubation period, the cultures were placed at 60 ºC, for 48 hours. After grinding, the aflatoxin B1 content of the inoculated corn was measured by HPLC. The results were evaluated with paired t-probe in Microsoft Excel (at $P<0.05$).

2.7 Inoculation of corn kernels with *Aspergillus flavus* for *in vitro* resistance testing of corn hybrids

A. flavus NRRL 11611 and an atoxinogenic and toxinogenic *A. flavus* isolates were inoculated on kernels of six different corn hybrids. 10-10 pieces of pretreated corn kernels, which were disinfected with 70% ethanol and washed with distilled water, were placed on Petri dishes, and every kernel was inoculated with 10µl 10^6/ml fungal spores in three replicates. Incubation was for seven days at 30 ºC in the dark (Luna-López et al., 2013; Rajasekaran et al., 2013). The growth and colonization ability of the fungus were investigated on the corn hybrids. The results were analyzed by paired t-probe using Microsoft Excel software (at $P<0.5$ significance level).

3. RESULTS

3.1 Nucleic acid-based analysis of the fungal isolates

The strains were collected between 2014-2015 from feeds and raw materials received at Central Laboratory of Agricultural and Food Products, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen. The *Aspergillus* genus was isolated from other molds on malate agar based on their common morphological characteristics (Diba et al., 2007; Varga et al., 2011). I selected mainly fungi with green-yellow conidia, which sometimes producing sclerotia, and showed rounded conidiophores and conidia in one or two series under microscopic examination. A total of 72 isolates from corn, corn-based feed, wheat, wheat flour was tested. I examined the potential presence of three genes *omtA*, *norA*, *aflR* from the gene cluster responsible for aflatoxin production using multiplex PCR (Geisen, 1996; Varga et al., 2011) (Fig. 1.).

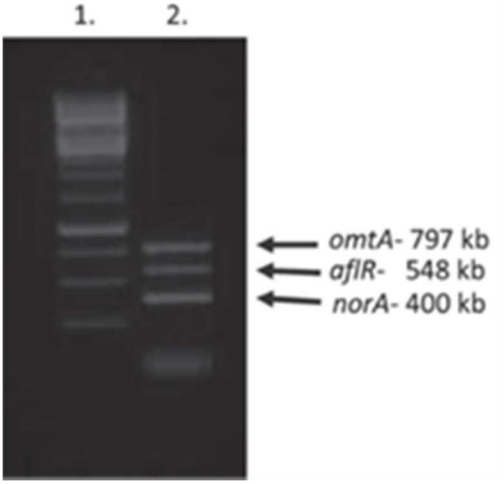

Figure 1. Typical results of multiplex PCR for amplification of the three genes 1. 1 kb DNA ladder, 2. result of multiplex PCR

In the studies, 33 of the 72 *Aspergillus* isolates contained at least one of the three genes from the aflatoxin gene cluster, and 17 of these fungi contained all the three genes. Only eight *Aspergillus* isolates of the 33 produced aflatoxin B1 on malt agar medium. In six of the toxin-producing isolates, the co-presence of *aflR*, *norA,* and *omtA* genes was detectable, but the *omtA*

in one isolate, and the others the *aflR* and the *omtA* genes could not be amplified; however, aflatoxin B1 production was detected, suggesting a modified sequence of the genes. *aflR* positivity was detected in 32 isolates, but only seven of them produced aflatoxin B1. In these isolates, the gene-cluster responsible for aflatoxin B1 production has probably been altered or mutated; therefore, the fungus is unable to produce aflatoxin B1.

Based on the DNA sequence between ITS1-4primer pairs, 22 of the 33 isolates are suggested to be *A. flavus,* four isolates are *A. tritici*, or previously used synonym *A. candidus,* and one isolate is *A. cristatus* or *A. amstelodami*. However, further studies are needed for correct species identification. In the case of *A. amstelodami,* it can produce the intermediate of aflatoxin biosynthesis, sterigmatocystin (Bukelskienė et al., 2006). Based on the studies, I detected the presence of all three genes belonging to the aflatoxin synthesis gene cluster for this isolate.

I selected two strains, an aflatoxin B1 producing and non-producing strains, based on the ITS sequences, are presumably *A. flavus* species and also the reference strain and their DNA segments obtained by PCR reactions with calmodulin primer pair were sequenced. The sequencing proved that the selected strains belonged to the species of *A. flavus*.

Based on the results, I can state that while the ITS sequence and the calmodulin gene sequence help in species determination and the PCR results of the aflatoxin gene cluster help to identify potential toxin production, none of the methods help in the selection of atoxigenic strains. It needs toxin measurement in addition to the induction of toxin production.

Based on the research, more than 40% of the isolates were potentially aflatoxin-producing ones. The genes can be expressed under appropriate environmental conditions, and the isolates can produce toxins, causing significant economic and human health damage for agriculture.

Table 2. Summary of the occurrence of the *omtA*, *norA* and *aflR* genes from the *Aspergillus* isolates (Kovács et al., 2017)

	aflR norA omtA	***aflR***	***norA aflR***	***aflR omtA***	***norA***
A. flavus (n=22)	13	6	2	-	1
non determined (n=11)	4	4	2	1	-
total (n= 33)	17 (51,5%)	10 (30,3%)	4 (12,1%)	1	1

3.2 Investigations on different culture conditions, stress effects

3.2.1 Investigation of *Aspergillus flavus* NRRL11611 strain on different culture media

I examined the reference *A. flavus* NRRL 11611 strain to produce aflatoxin B1 and sclerotia, and the rate of its mycelial growth on malt and cornmeal agar media. Cornmeal agar medium was made from corn flour or cornmeal with different fat content (1.7-7.2 m/m%), respectively, to which I also added glucose or salts. I used the cornmeal agar medium because it represents the natural environment well. The nitrogen content measured by the Kjeldahl method was 0.03 ± 0.01 (w/w)% (n = 3) for the media with a crude fat content of 4.05, and 7.2 (w/w) % and the other two media with lower crude fats contained 0.01 ± 0.01 (w/w) % (n = 3) nitrogen, so it was on the detection limit of the method. For all cornmeal agar media, the free D-glucose content was under the detection limit (0.001 w/w %). The polyphenol content was within a wide range for the four corn meal agar media studied (5.74–35.45 ± 0.5 mg GA equivalent/100g, n=5), while the flavonoid content falls within a smaller range (3.24-7.16 ± 0.3 mg CE equivalent/100g, n=5) and there was not a significant difference in the flavonoid content of cornmeal agar media containing 2.6 and 1.7% crude fat ($P < 0.05$).

I experienced that for cornmeal agar media containing 1.7 (w/w)% and 2.6 (w/w)% of crude fat, in which the nitrogen source was of organic origin and the carbon source was polysaccharide, the aflatoxin B1 production was not detected. However, in case of the higher fat content of the medium, the fungal growth was weaker, and sclerotium was not detected. However, using cornmeal with lower fat content, the mycelial growth was more robust and there was also sclerotia formation. Using cornmeal agar medium with a higher crude fat content (4.05 and 7.2 w/w%) as a medium, where an organic nitrogen source and a polysaccharide carbon source were also present, aflatoxin B1 production was detected, and I did not found a significant difference between the aflatoxin B1 concentrations of the two media. Mycelial growth and aflatoxin B1 production were optimal on malate agar medium, while aflatoxin B1 production and sclerotium production were also increased on corn meal-glucose agar medium with 2.6 (w/w)% crude fat content, but the vegetative growth was only weak. On cornmeal agar medium containing 2.6% (w/w) % crude fat content with inorganic salts, there was no aflatoxin B1 production detected, the mycelial growth was strong, and the sclerotium production was more intensive (Table 3). I concluded that aflatoxin production was induced by monosaccharides, as well as the higher content of fat, but the latter had less importance than

the carbon source. Vegetative growth was the most intensive on malate agar medium, medium growth was detected on cornmeal agar medium of the lowest and of the two highest fat content. I was able to obtain the highest level of sclerotium production using the inorganic nitrogen source, but I also experienced significant sclerotium production using the highest fat content in the cornmeal agar medium. Interestingly, where the sclerotium development was intensive, low or any aflatoxin B1 production was detected. It was proving that the inorganic nitrogen source, nitrate, inhibited aflatoxin production but not sclerotium development (Price et al., 2005).

Corn hybrids with high fat content are more advantageous to aflatoxin B1 production even if the antioxidant content of them is also high.

Table 3. The intensity of the growth, aflatoxin B1 production, and sclerotium development of *Aspergillus flavus* NRRL11611 surface cultures on different media (n=3, #SD < 20 %)**.**

Medium	**Carbon source**	**Nitrogen source**	**Mycelial growth**[##]	**AF B1 (ng/ml)**[#]	**Sclerotium development** [###]
MA*	Glucose and other organic	organic	++++	3620	-
KDA**1.7	poly-saccharide	organic	+++	-	+
KDA 2.6	poly-saccharide	organic	+	-	-
KDA 2.6+G***	glucose and poly-saccharide	organic	+	11.90	+
KDA 2.6+S****	poly-saccharide	organic and $NaNO_3$	++	-	+++
KDA 4.05	poly-saccharide	organic	+++	1.115	+
KDA 7.2	poly-saccharide	organic	+++	1.060	++

*MA: malate agar medium.

**KDA: cornmeal agar with different crude fat content.

***G: glucose.

****S: salts.

[##] Vegetative growth: +: maximum 25%; ++: 25-50%; +++: 50-75%; ++++: 75-100% Petri dish coverage

[###] Sclerotium production: +: maximum 10 sclerotium; ++: 10-100 sclerotium; +++: minimum 100 sclerotium on a Petri dish culture.

In addition to the increased crude fat content, the increase in polyphenol and flavonoid content could not inhibit the aflatoxin B1 production.

With correlation analysis, there was a strong correlation in the variables, but the strongest correlation was calculated between the crude fat and the flavonoid content of the medium (0.947) and between the crude fat and the aflatoxin B1 production (0.799).

3.2.2 Correlation between the number of spores used for the inoculation and the production of aflatoxin B1

I investigated the dependence of the concentration of aflatoxin B1 produced on malt or cornmeal agar medium on the number of spores used for the plate inoculation. The *A. flavus* NRRL 11611 reference strain was used for the experiment. I found that aflatoxin B1 production was the highest at the lowest applied amount of 100µl 10^1/ml spores on malt agar medium, although at higher spore numbers (at 10^3, 10^4, 10^5) there was no significant difference between the aflatoxin B1 concentrations produced ($P<0.05$). Moreover, the aflatoxin B1 production on cornmeal agar with 2.6 (m/m) % crude fat content was not detected at different spore concentrations.

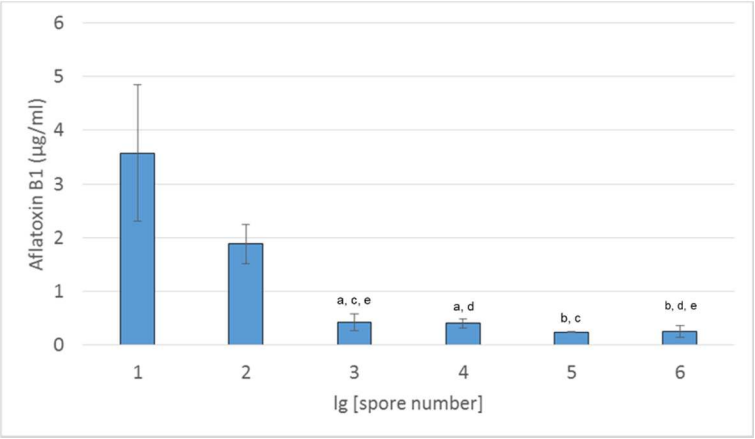

Figure 2 Differences in the concentration of aflatoxin B1 depended on the spore number of *Aspergillus flavus* NRRL 11611 on malt agar medium (n=3, SD=±10%). The same letter indicates the non-significant differences at the P<0.05 level.

3.2.3 Investigation on stress resistance of *Aspergillus flavus* isolates and the reference strain on cornmeal agar medium

In the case of the reference strain on the cornmeal agar medium, the osmotic stress influenced mainly the morphological, pigmentational changes, and spore production. Sclerotia were produced at all NaCl concentrations I used, which ensures the survival of the fungus under unfavorable environmental conditions.

In almost half of the isolates, osmotic stress also caused a change in pigmentation in fungal hyphae, while, in the case of two isolates, no morphological changes were observed, and in 8 isolates lower NaCl concentration inhibited the vegetative growth or spore production on cornmeal agar medium.

The reference strain produced high levels of sclerotium on cornmeal agar medium even at low concentrations of SDS, and 0.75 g/l SDS inhibited the vegetative growth completely. The SDS stress also had a similar effect on most of the isolates, the pigmentation of the conidiophores changed in 5 isolates, while, the spore production and growth changed in two isolates, which were not able to tolerate even lower SDS stress, while, in 5 isolates, this effect was only observed with 0.75 g/l SDS.

3.2.4 Investigation on stress resistance of *Aspergillus flavus* isolates and the reference strain on malt agar medium

During the examination of the *A. flavus* reference strain, I found its similar tolerance to the osmotic as well as SDS stress on malt agar medium as on cornmeal agar medium. NaCl was primarily caused changes in the pigmentation of hyphae and higher sclerotium production, and SDS reduced spore production at its higher concentrations and inhibited growth at 0.75 g/l.

From the aflatoxigenic isolates, only four were affected significantly in their morphological characteristics used by the lower NaCl concentration. In contrast, in the other isolates, a concentration of 2 M or higher NaCl concentrations had an effect on the fungal growth or spore production on malt agar medium.

Using SDS treatment on malt agar medium, I found that most of the isolates showed similar tolerance to this stress as the reference strain, three isolates changed in their morphology due to lower concentrations of SDS stress, while one isolate, the highest SDS concentration caused only low sclerotium production.

Overall, the resistance of the reference strain and the aflatoxigenic isolates was high against salt as well as SDS stresses on both cornmeal agar medium and malt agar medium, which may have great importance for plant protection.

3.3 Analysis of secondary metabolites produced on corn

The most considerable resistance against the application of aflatoxin-non-producing isolates is provided by the possibility of the production of other, lesser-known secondary metabolites. I wanted to investigate it in an in vitro experiment on corn kernels. In the research, I examined the presence of secondary metabolites from the non-aflatoxin-producing *A. flavus* inoculated on MV251 corn hybrid (Table 4), according to Uka et al. (2017). Among the non-aflatoxin-producing isolates tested, one isolate produced speradine F, another isolate produced 3-OH-speradine A, aspergilline D, and speradine I, and a third isolate produced detectable concentrations of aflatoxin B1 on the corn kernels (Table 4), thus, these could not be considered as atoxigenic strains. The other non-aflatoxigenic *A. flavus* isolates did not produce any of the secondary metabolites tested in HPLC-MS detectable amounts. The metabolites found were all CPA derivatives which possess less toxic effects than aflatoxins and, therefore, these are not so intensively tested compounds but may be relevant for human health due to their combined cumulative toxicological and long-term effects. In applications of atoxigenic fungi in biological

control, the knowledge on the secondary metabolite production of the fungus is crucial in the protection against aflatoxin contamination. Moreover, the examination of the responsible genetic background is of high importance. Hence, even if any secondary metabolite is produced, in the presence of the suitable genes, these genes may be activated under certain conditions, and it causes the production of harmful secondary metabolites.

Table 4. Summary: characterization of potentially non-aflatoxin-producing isolates

Isolates	**Produced compound (HPLC-MS)**	**Growth on MV 251 corn kernels**	**Presence of genes from the aflatoxin gene cluster (multiplex PCR)**		
			norA	*aflR*	*omtA*
7	-	weak	+	+	+
27	-	weak	-	+	-
28	-	weak	+	+	+
30	3-OH-speradine A, aspergilline D, speradine I	intense	+	+	+
33	-	intense	+	+	+
34	-	intense	-	+	-
37	speradine F	intense	+	+	+
44	-	weak	+	+	-
53	-	intense	+	+	+
61	-	intense	-	+	-
70	-	intense	+	+	-
72	aflatoxin B1	intense	-	+	-

3.4 Competition ability test

In biological control, *A. flavus* strain that does not produce aflatoxins can be used to reduce aflatoxin contamination in corn. Through the competitive ability of an atoxigenic strain, it can inhibit the growth of the toxigenic *A. flavus* and the production of its secondary metabolites. In the research, it was proved that the non-aflatoxin-producing *A. flavus* isolate also can reduce aflatoxin contamination in corn kernels (Figure 3), but this isolate was proven to produce speradine F resulted by later HPLC-MS studies. Therefore, the competition tests on other atoxigenic isolates with a toxigenic strain of *A. flavus* further required as well as *in vivo* field experiments to demonstrate the effectivity of aflatoxin-reduction of the isolates.

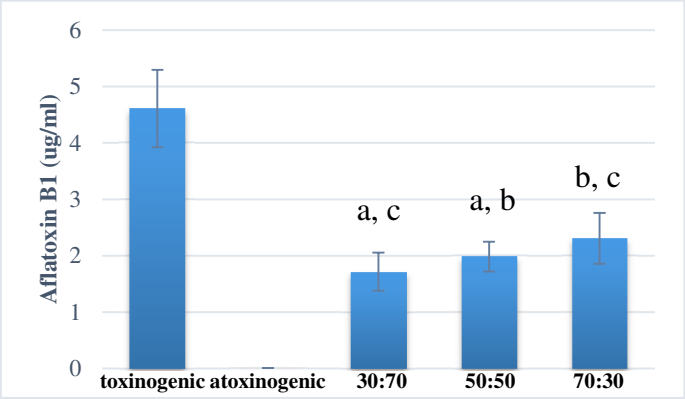

Figure 3. Aflatoxin B1 production on MV 251 corn kernels, toxigenic (9.), atoxigenic (37.) *Aspergillus flavus,* as well as co-infections of the two isolates in different ratios (n=3, SD<20%). The same letter indicates the non-significant difference at the P <0.05 level.

3.5 The resistance of corn hybrids and pathogenicity of *Aspergillus flavus*

The inoculation required for the corn kernel resistance experiment was performed according to *Rajasekaran et al. (*2013), which help to determine the sensitivity of each corn hybrid to *A. flavus*. In the research, I examined the ratio of the contaminated kernels of the different corn hybrids by the reference and the toxinogenic and non-aflatoxin-producing *A.*

flavus. The different corn hybrids showed different resistance and various contamination results against the different *A. flavus* strains. The colonization ability of the fungal isolates was similar, but it was different from that of the reference strain, which can be explained by the fact that the reference strain was not originated from corn but peanuts but could grow and produce aflatoxin B1 on corn.

There are currently any known corn hybrids with full resistance in this region, but there are corn hybrids with low, medium, and high sensitivity to the infection of each fungal species (Szabó et al., 2018).

Based on the studies, I was able to select a corn hybrid that behaved substantially similarly with the non-aflatoxin-producing and toxigenic *A. flavus* isolates in the *in vitro* experiment and, therefore, it can be used in further research.

4. NEW SCIENTIFIC RESULTS

1. **I assessed the aflatoxin-producing ability of endemic *Aspergillus flavus* isolates.** 72 *Aspergillus sp*. were collected from feeds and feed materials. The presence of three genes of the gene cluster responsible for aflatoxin B1 production (*aflR*, *norA*, *omtA*) was investigated using multiplex PCR. Of the 72 isolates, 33 contained at least one of the genes and were, therefore, potentially aflatoxin-producing. I identified the potentially aflatoxigenic isolates based on their ITS region using primers ITS1 and ITS4. Based on the results, 22 isolates proved to be potentially *A. flavus*. An atoxigenic and a toxigenic *A. flavus* isolate were also identified as *A. flavus* based on the calmodulin gene sequence.

2. **I described a relationship between corn crude fat content, the sclerotium production, and the aflatoxin production.** The aflatoxin-producing reference strain *A. flavus* produced aflatoxin B1 on cornmeal agar medium with high crude fat content and malt agar medium, and aflatoxin B1 production was also detected by adding glucose on cornmeal agar medium with lower crude fat content. The production of fungal sclerotium was mainly induced by high fat content and the use of inorganic nitrogen sources in the case of lower crude fat content. With intense sclerotium production, aflatoxin B1 production was not or only barely measurable.

3. **I identified the inverse relationship between the inoculum quantity and the aflatoxin B1 production under *in vitro* favorable conditions.** In the case of the lowest quantity of spores applied in the inoculation, the highest concentration of aflatoxin B1 production was observed on malt agar medium with the reference *A. flavus* strain. In this case, the size of the fungal colonies was the highest, and the mycelial coverage of the Petri dish was the lowest.

4. **I characterized the stress tolerance of the reference and the isolated *Aspergillus flavus* strains.** The reference *A. flavus* strain responded to changes of pigmentation against osmotic stress on malt agar medium and cornmeal agar medium, and its sclerotium production was also increased on malt agar medium. While SDS, which damages cell membranes, increased the sclerotium production at low concentrations and decreased vegetative growth at higher concentrations. About half of the *A. flavus* isolates that I collected responded similarly to osmotic stress on cornmeal agar medium as the reference strain; however, in some isolates, only higher concentrations of NaCl inhibited the growth or the conidiospore production. While on malt agar medium, lower osmotic stress caused morphological changes only in four isolates, and the other isolates were not affected in their growth or conidiospore formation. Two isolates on cornmeal agar medium and three of them on malt agar medium responded even to lower

SDS stress with reduced spore production and vegetative growth, while most of the isolates behaved similarly to the reference strain.

5. **I identified isolates potentially without toxic metabolite production according to the HPLC-MS technique.** Nine atoxigenic *A. flavus* isolates were found that did not produce any of the 30 secondary metabolites tested when inoculated on corn kernels.

6. **In *in vitro* experiment, the aflatoxin-non-producing isolate successfully inhibited the growth and toxin production of the toxigenic isolate.** I have demonstrated that the characterized and applied atoxigenic *A. flavus* isolate can reduce the aflatoxin B1 contamination caused by the toxigenic *A. flavus* isolates in corn kernels.

7. **I tested the growth of toxigenic and non-aflatoxin producing *Aspergillus flavus* on different corn hybrids.** By investigating six different corn hybrids, I successfully isolated hybrids with different resistance and sensitivity to *A. flavus* fungal infection, as well as found a hybrid that was similarly infected by atoxigenic and toxinogenic isolates.

5. RESULTS UTILIZABLE IN PRACTICE

1. The aflatoxigenic *A. flavus* isolates that I collected showed high resistance to osmotic as well as cell membrane stress, which may be distinguished for the plant protection.

2. I showed that the resistance of the studied corn hybrids is different to *A. flavus*, as well as to the different toxigenic, atoxigenic, and reference *A. flavus*. I found a hybrid with similar resistance to the toxinogenic and atoxigenic *A. flavus* isolates to support the further *in vivo* experiments.

3. I identified *A. flavus* isolates that did not produce aflatoxin B1 in corn and did not produce any of the tested 30 secondary metabolites examined by HPLC-MS. Thus, these can be applied successfully under field conditions as biocontrol agents against aflatoxin B1 contamination in corn.

4. In the competition test, I proved that an *A. flavus*, which did not produce aflatoxin B1, inhibited aflatoxin B1 production in corn; thus, it can be used effectively as a biocontrol.

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7. LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

1. **Kovács, S.**, Pusztahelyi, T.: Investigation of polyphenol resistance of Aspergillus flavus on Cornmeal media.
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