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Investigation of Polyphenol Resistance of *Aspergillus flavus* on Cornmeal Media

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Cornmeal agar (CMA) is a good to model natural conditions (low C and N, high antioxidants, crude fat) for phytopathogenic fungi. Different CMA media was prepared to model the maize kernel as growth environment for *Aspergillus flavus*, where stress resistance and aflatoxin B1 (AFB1) production were tested. The CMA medium with high polyphenol and low fatty acid content did not support the mycelial growth and high AFB1 production but the sclerotia development of the cultures. High fatty acid content in the CMA exceeded the inhibitory effect of antioxidant polyphenols of corn and low concentration of AFB1 was detected. Glucose supplement of CMA induced AFB1 production proving the need for free carbon source for the secondary metabolite pathway. The tolerance of the fungus against salt and cell membrane stress was lowered on CMA. At higher fatty acid concentration, the aflatoxin B1 production cannot be hindered by the natural antioxidants and that is important in selection of resistant corn hybrids.

Keywords: Aflatoxin B1, *Aspergillus flavus*, polyphenol, flavonoids, stress tolerance, cornmeal.
Abbreviations: AFB1, aflatoxin B1; MA, malate agar; CMA, cornmeal agar.

Mycotoxin production by the microscopic filamentous fungi has always caused a crucial problem worldwide especially in monocultures and in storage crops. *Aspergillus flavus, A. parasiticus* (Mayer et al., 2003; Schmidt-Heydt et al., 2008) and other *Aspergilli,* besides some *Rhizopus* strains (Erdogan, 2004; Cary et al., 2005; Varga et al., 2009), are the well-known producers of the carcinogenic secondary metabolites, aflatoxins (AFs). Aflatoxin B1 (AFB1), is the most toxic compound known that is commonly controlled worldwide especially in oily seeds and fruits. The physiological role of AFs is not clear. However, there is an evidence that they have insecticide properties (Grintzalis et al., 2014).

Preharvest contamination of the crops with AFs is usual, but the fungi also cause AFB1 spoilage post-harvest resulting in significant economic losses. The optimum temperature of the AF production is about 32–38 °C. Climate changes, characterized by the decrease in summer precipitation and increase the average temperature worldwide (the global temperature is expected to increase by between + 2 °C and + 5 °C; Medina et al., 2014), significantly enhances the danger of AF contamination.

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Recently, our studies revealed the importance of the aflatoxin measurements of crops (Kovács and Pusztahelyi, 2017). Among the yellow *Aspergillus* isolates that were gained from crop samples, higher than 40% of the fungi were able to produce aflatoxins (Kovács and Pusztahelyi, 2017) based on the presence of the characteristic AF cluster genes. However, the nucleic acid-based detection gave only a suggestion on the potentially aflatoxigenic strains and the intensity of the mycotoxin production highly depends on the environmental conditions.

Environmental stresses, sources of abiotic stress like drought or heat stress, the effect on the plant-fungal interactions (e.g., Fountain et al., 2014) are all has an impact on mycotoxin production and mycelial growth. Fungal genes involved in stress-related responses, especially in oxidative stress, are numerous in phytopathogenic fungi (see, e.g., FSRD: Fungal Stress Response Database; Karányi et al., 2013) and fungal toxins often trigger these stress-related responses. Potentially, fungal oxidative stress and regulation of AFB1 production have a positive feedback control (Huang et al., 2009) as the production of aflatoxins seemed to be favored by an oxidative environment; e.g., the oxidative stress was reported to induce aflatoxin accumulation (Jayashree and Subramanyam, 2000). In plants, polyphenols are involved in defense against UV radiation and pathogens. The mechanisms of antioxidant action can include suppression of fungal reactive oxygen species (ROS) formation, scavenging ROS, and upregulation or protection of antioxidant defenses, and the action of plant flavonoids involves most of these mechanisms.

In the present paper we studied the effect of the plant polyphenols on mycotoxin production. We prepared different environment (with different polyphenol, fatty acid, glucose, NaCl, SDS content) in cornmeal agar applied as a natural medium and investigated how growth, development, AFB1 production and sclerotium development of *Aspergillus flavus* was influenced.

Materials and Methods

Cultivation of Aspergillus flavus

Aspergillus flavus NRRL11611 was streaked onto malate agar (MA) medium [20 g l^{-1} glucose, 10 g l^{-1} malate extract, 5 g l^{-1} yeast extract, 15 g l^{-1} agar] and cultivated for five days at 30 °C in dark.

Preparation and characterization of culture media

Coarsely milled corn products characterized with different chemical composition were added to distilled water (15 g l^{-1}) and boiled for 30 min. The suspensions were filtered through microfibre clothes, the filtrates were mixed with 20 g l^{-1} agar, and afterward, the prepared cornmeal agar media (CMA) were autoclaved. The antioxidant polyphenol content of the sterilized agar media was determined by Folin-Ciocalteu method and given as gallic acid equivalent (GAE) (Kaur and Kapoor, 2002). Flavonoid (flavone/flavonol) content was determined per Chang et al. (2002) as catechin equivalent (CE). The crude fat content of the culture media was gained by petrol ether extraction by the modified Randall method in Soxtec extractor (TECATOR). Nitrogen content was measured by the Kjeldahl

method after total hot acid disruption of the samples and distillation of ammonium (VELP Scientifica). The D-glucose content of the media was detected spectrophotometrically with the Glucose Kit of Megazyme. CMA media were named after the crude fat content of the media: 1.7 m/m% (CMA1.7), 2.6 m/m% (CMA2.6), 4.05 m/m% (CMA4.05), 7.2 m/m% (CMA7.2).

The CMA2.6 medium was also supplemented with 3 g l^{-1} $NaNO_3$, 0.5 g l^{-1} $MgSO_4 \times 7H_2O$, 0.5 g l^{-1} NaCl, 0.01 g l^{-1} $FeCl_3 \times 7H_2O$ (CMA2.6 + S) or with 20 g l^{-1} glucose (CMA2.6 + G).

SDS and salt stress resistance of the surface cultures

The fungal strains were maintained in surface cultures on MA plates, and the conidiospores were collected by washing the mycelial mat with sterile 1% TWEEN-20 solution. The concentration of the spores was determined microscopically in a hemocytometer.

The MA and CMA2.6 plates, both were supplemented with stress agents of different concentrations: NaCl (up to 2.5 M), or SDS (up to 75 g l^{-1}), were inoculated with 10^2 spores and incubated at 30 °C in the dark. The growth and development of the strains were evaluated after 5–7 days of inoculation.

Measurement of aflatoxin B1

The agar medium together with the one-week-old surface culture (inoculated with 10^1–10^6 spores) was collected in sterile Stomacher homogenizer bag and, with 10 ml chloroform, it was homogenized in Stomacher homogenizator (Masticator, IUL Instruments) for 2 min, and the process was repeated twice. 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 Millex-GV 0.22 μm filter (Merck-Millipore).

The AFB1 content of 20 μl samples was determined by HPLC technique on Hibar 125-4 Lichrospher 100RP-18 (5 μm) column with 1 ml min^{-1} flow rate of the mobile phase (methanol: water, 45:55) and AFB1 was detected by a fluorescence detector at ex360nm, em440nm. Biopure Aflatoxin B1 standard (Romer Labs) solution was applied to the column.

Results

Effect of media composition on development and aflatoxin B1 production

The growth of the mycelia, aflatoxin production, and sclerotium development was investigated on different media supplemented with organic and inorganic N sources and glucose or complex carbon sources (Table 1). To model natural conditions, we inoculated cornmeal agar media (CMAs) and we compared them to cultures on the known malate agar (MA) medium.

The CMA media presented different crude fat and antioxidant content for the cultures. We prepared four different type of media with a wide range of crude fat (1.7–

7.2 ± 0.3 m/m%, n = 3) and polyphenol (5.74–35.45 ± 0.5 mg GA equivalent 100 g^{-1}, n = 5) content. While, considering the flavonoid content (3.24-7.16 ± 0.3 mg CE equivalent 100 g^{-1}, n = 5) the range was much smaller (Fig. 1). The free glucose content was under the detection limit (0.001 m/m%) in any of these media. Meanwhile, the nitrogen content was measurable (0.03 ± 0.01 m/m%, n = 3) in the CMA4.05 and CMA7.2 media, while in the CMA2.6, CMA1.7 media it was measured at the detection limit (0.01 m/m%) by the Kjeldahl method.

The mycelial growth was strong in cultures on MA, while was much weaker on CMA agar media. Meanwhile, the development of the sclerotia was more intensive on the CMA plates (Fig. 2, Table 1). *A. flavus* NRRL11611 strain produced a detectable amount of AFB1 toxin on the MA, CMA4.05, CMA7.2, and glucose supplemented CMA2.6 (CMA2.6 + G) plates (Table 1, Fig. 1). On CMA2.6, CMA 1.7, and the salt amended CMA2.6 (CMA2.6 + S) media (Fig. 2) the AFB1 production was not detected. The highest values of AFB1 (3.6 μg/ml) were detected on the MA medium (Table 1). Interestingly, where the sclerotium development was intensive, low or any AFB1 production was detected.

Table 1

Intensity of growth, aflatoxin B1 production and sclerotium development of *Aspergillus flavus* NRRL11611 on different surface cultures: MA, malate agar medium; CMA1.7, CMA2.6, CMA 4.05, CMA7.2 are cornmeal agar media; CMA2.6 + S, cornmeal agar medium + salts. CMA2.6 + G, cornmeal agar medium + glucose

Medium	Carbon source	Nitrogen source	Mycelial growth	AFB1 (ng/ml)*	Sclerotium development
MA	Glucose and other organic	organic	+ + + +	3620	–
CMA1.7	Polysaccharide	organic	+ + +	–	+
CMA2.6	Polysaccharide	organic	+	–	–
CMA2.6 + G	Glucose and polysaccharide	organic	+	11.90	+
CMA2.6 + S	Polysaccharide	organic and $NaNO_3$	+ +	–	+ + +
CMA4.05	Polysaccharide	organic	+ + +	1.115	+
CMA7.2	Polysaccharide	organic	+ + +	1.060	+ +

*mean ± 10% R

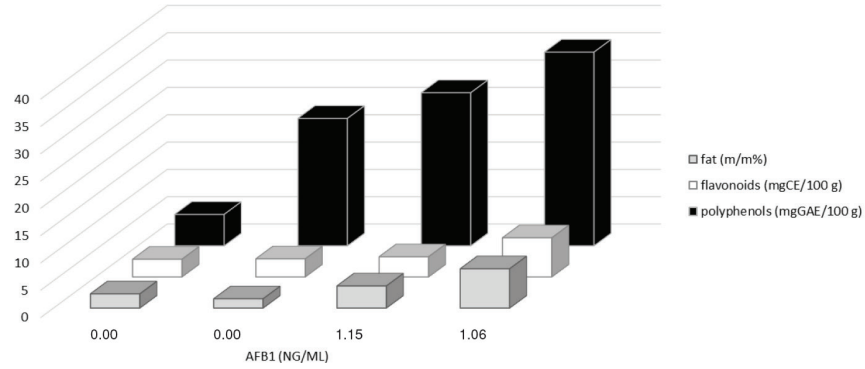

Fig. 1. Aflatoxin B1 production dependence on polyphenols, flavonoids and crude fat content in the cornmeal agar media

Stress resistance of cultures in MA and CMA2.6 medium

The stress resistance of *A. flavus* NRRL11611 was impressively high on the MA surface cultures but weaker on CMA2.6. In surface cultures on MA, NaCl supplemented up to 2.5 M concentration did not hinder the growth of *A. flavus* NRRL 11611. Only some changes such as an insignificant decrease in biomass production, depigmentation of the conidiophores were detected. Meanwhile, above 0.5–0.75 g l^{-1} SDS, the fungal germination was inhibited, and developmental differences like decreased conidiogenesis, reduced pigmentation of the conidia or the higher presence of sclerotia were detected. The AFB1 production of *A. flavus* NRRL 11611 was not hindered even by 1 M NaCl

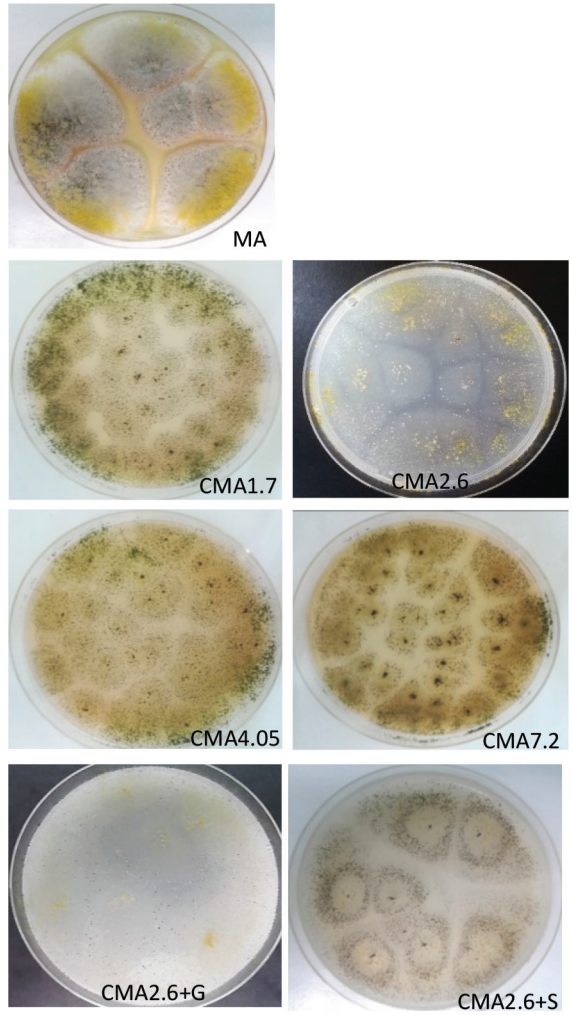

Fig. 2. Growth and development of *Aspergillus flavus* NRRL11611 on different surface cultures: on malate agar medium (MA); on cornmeal agar media with different crude fat content: 1.7 m/m% (CMA1.7), 2.6 m/m% (CMA2.6), 4.05 m/m% (CMA4.05), 7.2 m/m% (CMA7.2); on cornmeal agar medium + glucose (CMA2.6 + G); on cornmeal agar medium + salts (CMA2.6 + S)

and 0.1 g l^{-1} SDS on MA plates as 2.52 μg ml^{-1} and 1.13 μg ml^{-1} AFB1 were produced, respectively.

On CMA2.6 medium, the development of yellow pigmented conidiophores and the production of sclerotia were detected up to 1.5 M NaCl supplement concentration, in contrast to higher concentrations, where the pigmented conidiophores were not developed. In the cultures, which supplemented with 0.05 g l^{-1} or 0.1 g l^{-1} SDS, a high number of sclerotia were observed after five days of incubation. While, on plates with 0.5 g l^{-1} SDS supplement, conidiophore production of the cultures was reduced. Moreover, by increasing the SDS concentration to 0.75 g l^{-1}, no fungal outgrowth was detected.

Discussion

Malate agar (MA) medium is a standard medium which supported growth and asexual development of Aspergilli. From the point of the mycotoxin production, MA, which supplied the fungal cultures both with organic N and glucose in high concentration, induced the biosynthesis of aflatoxin B1 (AFB1), a secondary metabolite. It was shown, that the biosynthesis of AFB1 is dependent on glucose supply, which should have been higher than 0.1 M (Wiseman and Buchanan, 1987). Its concentration affected the production of the precursor of AFB1, sterigmatocystin, through modulating the light-dependent subcellular localization of VeA (velvet A protein in Aspergilli) and other components of the velvet complex (VelB-LaeA-KapA) (Calvo, 2008; Bayram et al., 2008).

It was concluded that N content also needed to switch on the AF biosynthetic pathway. Filamentous fungi can use several compounds as sole nitrogen sources, but preferentially use energetically favored nitrogen such as NH^{4+} and glutamine. Less easily assimilated nitrogen sources such as nitrate, amines, amides, purines, and pyrimidines can be used in the absence of the compounds mentioned above (Wong et al., 2008). Nitrate as sole N source was detected to inhibit AFB1 production (Kachholz and Demain, 1983), while the organic nitrogen forms increased the AFB1 production in *A. flavus* and *A. parasiticus* (Payne and Hagler, 1983). Therefore, the MA medium was an optimal environment because of the high glucose and organic N concentration which supported the mycelial growth and, in the late growth phase, the secondary metabolite production. Commercial CMAs are usually applied as strain maintenance media but there were no data on polyphenols and flavonoids or fatty acid content of these media. Our prepared CMA culture media were characterized and contained relatively high natural antioxidant and fatty acid content, low free glucose and N.

Mahoney and Molyneux (2010) concluded that plant-derived antioxidants usually diminish aflatoxin formation without affecting fungal growth. In contrast, degraded growth even under glucose supplementation was characteristic to the cultures on CMAs.

Salt and SDS stress resistance were remarkable both on CMA and MA media but the applied stresses were more tolerable on MA medium. Natural isolates of the aflatoxigenic fungi also have a high tolerance against salt and membrane stresses (Kovács's personal communication) that is of interest considering plant protection.

In contrast to MA, the high antioxidant content and the low free available sugar content of the CMAs highly induced sclerotia development, the resistant structures which

designed to withstand harsh environmental conditions (Coley-Smith and Cooke, 1971) on fields.

Moreover, at high fatty acid content, measurable AFB1 was detected on CMAs. It was known that AFB1 production depended on crude fat content (Howlett, 2006). We concluded that high fat content (from about 4 m/m%) and low N content resulted in the induction of the aflatoxin biosynthesis even when the fungal cultures faced with high antioxidant concentration. The suggested reason was that fungi derive acetyl-CoA from fatty acids for the biosynthesis of AFs (Howlett, 2006). Therefore, at low free carbon sources, what was the case in CMAs, high amount of fatty acids also can be an inducible environment for the AFB1 toxin production (Fabbri et al., 1983).

Under field conditions starch hydrolytic activities of maize, which are induced by the fungus, support the pathogenic *A. flavus* with enough free glucose (Dolezal et al., 2014) for the growth. Maize hybrids with high fat content are better targets to AFB1 contamination even if the antioxidant content of them is also high. Meanwhile, sclerotium production induced by the low nutrient, provide survival for the strains on fields. That fact is a critical knowledge as the climate change can increase the possibility of the contamination of the crops with aflatoxin producing and stress resistant fungi (Medina et al., 2014) CMA media gave the possibility to investigate toxin producing fungi under "natural-like" environment and this kind of application was new to the best of our knowledge.

Conflict of interests

The authors declare no conflict of interest.

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