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ARTICLE

# Investigation of aflatoxin production in three different *Aspergillus* species

**Barbara Tóth-Buella[1], Levente Horváth[2], László Kredics[1], Csaba Vágvölgyi[1], Tamás Papp[1], Tibor Bartók[2], Mónika Varga[1,†], András Szekeres[1,†,*]**

[1]Department of Biotechnology and Microbiology, Faculty of Science and Informatics, University of Szeged, H-6726 Szeged, Hungary
[2]Fumizol Ltd., H-6725 Szeged, Hungary

**ABSTRACT** Mycotoxins are secondary metabolites produced by molds, particularly by *Aspergillus* species, with their production influenced by various environmental and other factors. The growth and physiology of fungi are affected by factors such as temperature, water activity, and time. The aim of this study was to identify the best aflatoxin-producing *Aspergillus* strain among known mycotoxigenic species and isolates and to fine-tune specific cultivation conditions to achieve the highest aflatoxin yield. For this purpose, aflatoxin production was tested in isolates belonging to *Aspergillus pseudotamarii* (1), *A. minisclerotigenes* (1), and *A. parasiticus* (3). The presence and quantity of aflatoxins were determined by HPLC with fluorescence detection. Among the examined strains, *A. parasiticus* SZMC 22361 proved to be the most suitable toxin-producing strain. Our findings suggest that the optimal conditions for aflatoxin production by this isolate on a maize substrate involve a water-to-substrate ratio of 1:1 (m/m) over a production period of approximately one week. **Acta Biol Szeged 68(1):38-45 (2024)**

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***Corresponding author**
E-mail: andras.j.szekeres@gmail.com

## Introduction

Members of the *Flavi* section within the genus *Aspergillus* are among the most intensively studied fungi because they produce carcinogenic aflatoxin (AF) mycotoxins in agricultural products. AFs are among the most significant and toxic mycotoxins affecting both animal and human health (Peraica et al. 1999; Hussein 2001). Several chemical variants exist, with the most important being aflatoxins B1, B2, G1, and G2 (AFB1, AFB2, AFG1, AFG2), of which AFB1 is the most toxic. In the field or during storage, high temperatures and humidity promote the growth of toxigenic fungi and the accumulation of mycotoxins in foodstuffs (Lahouar et al. 2016). When accumulated in the body, these toxins can have mutagenic, carcinogenic, and teratogenic effects, as well as cause liver damage (Bhat et al. 2010). Due to the extreme toxicity of aflatoxins, strict regulations have been established worldwide, including in the European Union, to limit AF levels in food and animal feed (Commission Regulation (EU) 401/2006; Commission Regulation (EU) 1881/2006). To ensure effective compliance with these regulations, commercially standardized toxins are required for analytical testing (Steiner et al. 2024). These are typically obtained through the purification of compounds from the fermentation products of toxigenic fungi (Endre et al. 2023). Therefore, selecting the most suitable producer strain is essential for obtaining significant amounts of these substances in their pure forms as reference standards (Endre et al. 2023).

Within the genus *Aspergillus*, the most significant AF producers are *A. flavus* and *A. parasiticus*. *A. flavus* typically produces AFB1 and AFB2, while *A. parasiticus* also synthesizes AFG1 and AFG2 in addition to AFBs. *A. parasiticus* isolates usually produce high levels of AFs, while non-aflatoxigenic strains are rare (Tran-Dinh et al. 1999). *Aspergillus pseudotamarii*, which produces AFB1 and AFB2, has been isolated from crops and has also been reported to cause eye infections (Ito et al. 2001; Ehrlich et al. 2003; Baranyi et al. 2013). In addition to AFs, this species produces various secondary metabolites, including cyclopiazonic acid, kojic acid, aspirochlorin, altersolanol, several speradines, fumifungin, sphingofungins B-D, tenuazonic acid, phytosphingosine, ustilaginoidin C, canadensolide, ditryptophenaline, and ergokonin B, among others (Ito et al. 2001; Varga et al. 2011; Frisvad et al. 2019; Wang et al. 2021). *Aspergillus minisclerotigenes* is another highly toxigenic species, first described in 2008 (Pildain et al. 2008), and later reported from Eastern Kenya (Probst et al. 2012). Although certain isolates were previously classified as *A. flavus* (Geiser et al. 1998, 2000), *A. flavus* isolates produce only AFBs, whereas *A. minisclerotigenes*

†These authors contributed equally to this work, M.V. and A.Sz. share last authorship.

isolates can produce both AFBs and AFGs (Klich 2007).

The main factors influencing fungal survival, growth, and ecophysiology are temperature, water activity, and time, although fungi have adapted to endure extreme biophysical conditions (Magan et al. 2003; Bosch et al. 2021; Lloyd 2021; Hallsworth 2022). *Aspergillus* species are ubiquitous in soil, where their growth is promoted by heat and humid environmental conditions. The production of AFs is influenced by various environmental and ecological factors, such as temperature, water activity, pH, light, and substrate type (Schmidt-Heydt et al. 2009; Gizachew et al. 2019). Among these, water activity and temperature play key roles during storage, significantly affecting fungal growth and mycotoxin production (Liu et al. 2017). For *A. flavus*, extensive research has been conducted on the effects of cultivation parameters on AF production. Optimal growth conditions for these isolates on peanut seeds have been reported between 28-40 °C and 0.94-0.99 *aw* for temperature and water activity, respectively. At temperatures below 20 °C or water activity values below 0.90, fungal growth was completely absent. The most favorable conditions for AFB1 production were found to be between 25-33 °C and 0.92-0.96 *aw*, with the highest AFB1 levels measured at 28 °C and 0.96 *aw* (Liu et al. 2017). Thus, the optimal conditions for growth and aflatoxin production differ for the examined *A. flavus* strain.

Beyond temperature and water activity, AF production in *A. flavus* is also influenced by food substrates and nutrient composition (Gallo et al. 2016). For *A. parasiticus* strains, storage temperature affects AFG1 production more than moisture content, while neither incubation temperature nor moisture content significantly impacts AFG2 production (Davis et al. 1968; Akinola et al. 2021). Temperature and storage duration influence AFB2 production more than moisture content, while the interaction between moisture content and incubation duration does not significantly affect total aflatoxin production in wheat flour (Akinola et al. 2021). Additionally, wet-harvested edible nuts provide suitable conditions for fungal growth and AFB1 production, with optimal conditions reported at 25 °C and 0.96 *aw* for walnuts and 30 °C and 0.96 *aw* for peanuts (Bukhari et al. 2023). In contrast, limited literature is available on the AF production of *A. pseudotamarii* and *A. minisclerotigenes* under different cultivation conditions.

The highest concentrations of AFs have been detected in peanuts, but they are also common in cottonseed, almonds, pistachios, chickpeas, and chickpea-based products (Romero Donato et al. 2022), as well as in soybeans, rice, millet, coffee, corn, and other cereals (Varga et al. 2009). Monitoring AF contamination is crucial in the food industry, leading to the development of increasingly sensitive detection methods over the years using various instrumental techniques (Schincaglia et al. 2023; Rakk et al. 2023).

Solid substrates commonly used for AF production include feed and food matrices such as ground coffee (Soliman 2002), rice, wheat, triticale (Bilotti et al. 2000), corn, and corn husks (Shotwell et al. 1980). Cultivation on grains can model infestations in storage facilities and may help map toxin production, particularly when key growth factors are altered. Another critical factor affecting fungal growth is cultivation time (Gqaleni et al. 1997).

By modifying key parameters involved in aflatoxin biosynthesis, toxin production can be either inactivated or enhanced. Therefore, determining the optimal temperature and water activity values for the growth and AF production of *Aspergillus* isolates is essential. In this study, the AF production capabilities of one *A. pseudotamarii*, one *A. minisclerotigenes*, and four *A. parasiticus* isolates were investigated to identify the best producer strain and the most suitable cultivation conditions.

## Materials and Methods

### *The examined strains*

Initially, strains *A. pseudotamarii* SZMC 25517, *A. minisclerotigenes* SZMC 22438, and *A. parasiticus* SZMC 24773 were tested for AF production, then additional *A. parasiticus* strains (SZMC 22727, SZMC 22728 and SZMC 22361) were also selected based on the results of the pilot examinations. All examined strains derived from the Szeged Microbiology Collection (http://szmc.hu/).

### *Applied cultivation parameters*

For the inoculation of the solid substrates, the strains were pre-cultivated on potato dextrose agar (PDA, VWR International, Hungary) at 28 °C for 7 days. For the toxin production, maize was used as substrates grounded into small pieces (Mill 3310, Perkin Elmer, USA) to facilitate fungal growth. For the cultivations, 10 g of maize was placed in 100 ml Erlenmeyer flasks and 10 g water was poured on them. The flasks were then sterilized on two consecutive days using an autoclave at 100-120 °C and allowed to cool after vigorous shaking. Agar blocks with 1 cm in diameter were removed from the edge of fungal colonies using a cork borer and placed into the Erlenmeyer flasks containing the sterilized substrates. The fungal cultures were incubated in dark at 28 °C for one week.

To study the effects of water content, two additional water content was also tested (8 g and 12 g).

The influence of the cultivation length on the AF production was monitored for one, two and three weeks.

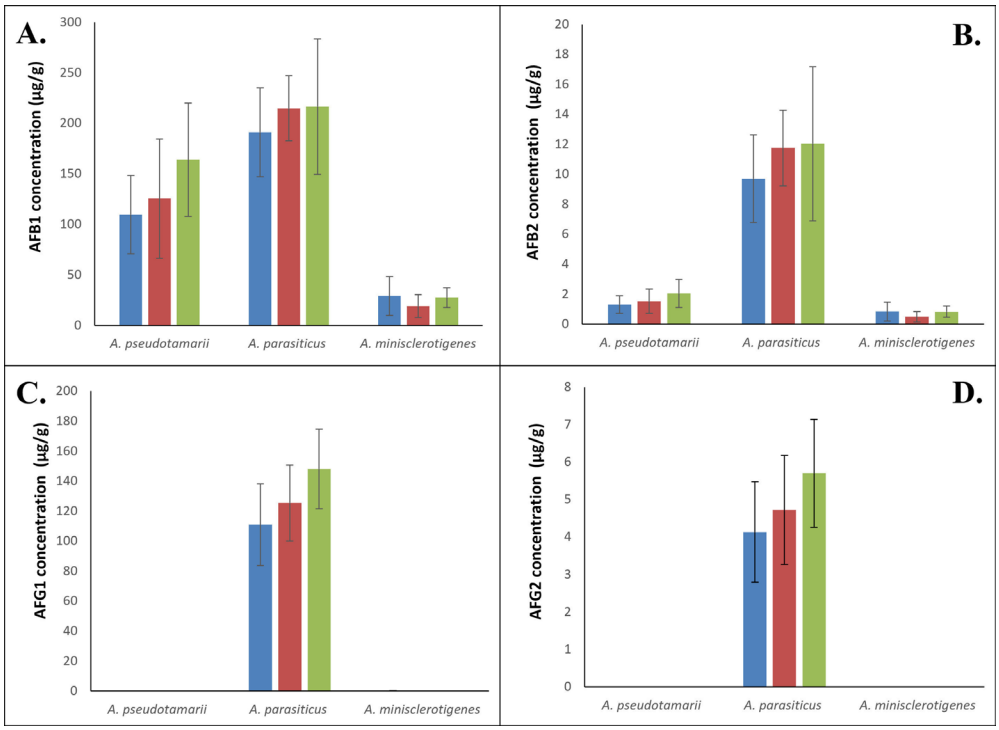


**Figure 1.** Production of AFB1 (A), AFB2 (B), AFG1 (C) and AFG2 (D) of *A. pseudotamarii* SZMC 25517, *A. parasiticus* SZMC 24773 and *A. minisclerotigenes* SZMC 22438 after one week of fermentation on ground corn supplemented with 8 g (blue), 10 g (red) and 12 g (green) water during the preparation of the medium.

### *Sample pretreatment*

After the incubation period of the cultures, the extraction was carried out with 40 ml of methanol (VWR International, Hungary) from each flask. The samples were then allowed to stand overnight and were extracted with overhead shaker (Rotax 6.8, Velp Scientifica-Lab Solutions, Italy) for two hours. The samples were then centrifuged for 10 min at 10.000 rpm (Sorvall RC 6 Plus, Thermo Fisher Scientific, USA). After that, 40 ml hexane (VWR International, Hungary) was added to the samples (in 1:1 ratio) and the fatty components extracted into the upper phase, which was discarded. The defatted methanolic sample was supplemented with 40 ml of chloroform (VWR International, Hungary) and water was added until two phases formed and extracted in two repetitions. The lower organic phases were dried over $MgSO_4$, membrane-filtered (0.2 µm PTFE filter, Whatman, UK) and evaporated to dryness. The dried extract was dissolved in 1 ml methanol and transferred into a HPLC vial prior to HPLC injection.

### *Analytical measurements*

Analyses were performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a DGU-20A5R degasser, an LC-20AD binary pump, a SIL-20A autosampler, a CTO-10ASvp column thermostat, an RF-20A fluorescence detector (FLD), and a CBM-20A system controller. The data were acquired and evaluated with Class VP ver. 6.2 software (Shimadzu, Kyoto, Japan). The separation of AFs was performed on an injected sample volume of 3 µl in a Phenomenex Kinetex C18, 100 mm × 4.6 mm, 2.6-µm column (Phenomenex, California, USA) with the mobile phase comprising water (A), methanol (B), and acetonitrile (C) combined in an A/B/C ratio of 60/30/20 by volume. The flow rate was 0.5 ml/min, and the column temperature was maintained at 30°C. For fluorescent detection, $\lambda_{excitation}$ = 350 nm and $\lambda_{emission}$ = 450 nm were applied.

### *Statistical analysis*

All fermentations were carried out in three repetitions and after the chromatographic measurements the averages and the standard deviations were determined. The statistical analyses were performed using GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA, USA, 2016). The significant differences were determined by one-way analysis of variance with Bonferroni's multiple comparison tests.

**Figure 2.** Fermentation of *A. parasiticus* SZMC 24773 on ground corn supplemented with 8 g (A), 10 g (B) and 12 g (C) water during the preparation of the medium.

## Results and discussion

### *Effects of water amount on the AF production of the examined species*

Initially, three strains from different species within *Aspergillus* section *Flavi* (*A. pseudotamarii* SZMC 25517, *A. minisclerotigenes* SZMC 22438, *A. parasiticus* SZMC 24773) were tested for AF production at different water content levels (Fig. 1). Under the applied cultivation conditions, the *A. parasiticus* strain produced the highest amounts of all AFs across all moisture conditions. The *A. pseudotamarii* and *A. minisclerotigenes* strains exclusively produced AFB1 and AFB2 mycotoxins in all fermentation setups.

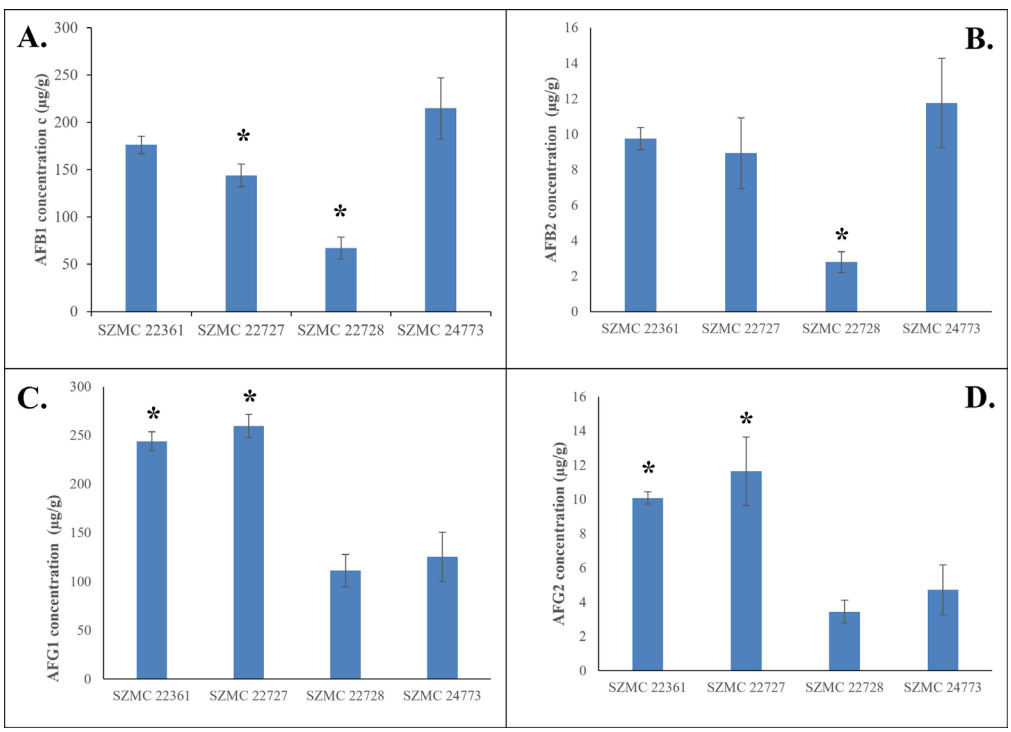


**Figure 3.** Production of AFB1 (A), AFB2 (B), AFG1 (C) and AFG2 (D) of *A. parasiticus* strains SZMC 22361, SZMC 22727, SZMC 22728 and SZMC 24773 after one week of fermentation. The asterisks show the significant differences ($P \leq 0.05$) from *A. parasiticus* SZMC 24773.

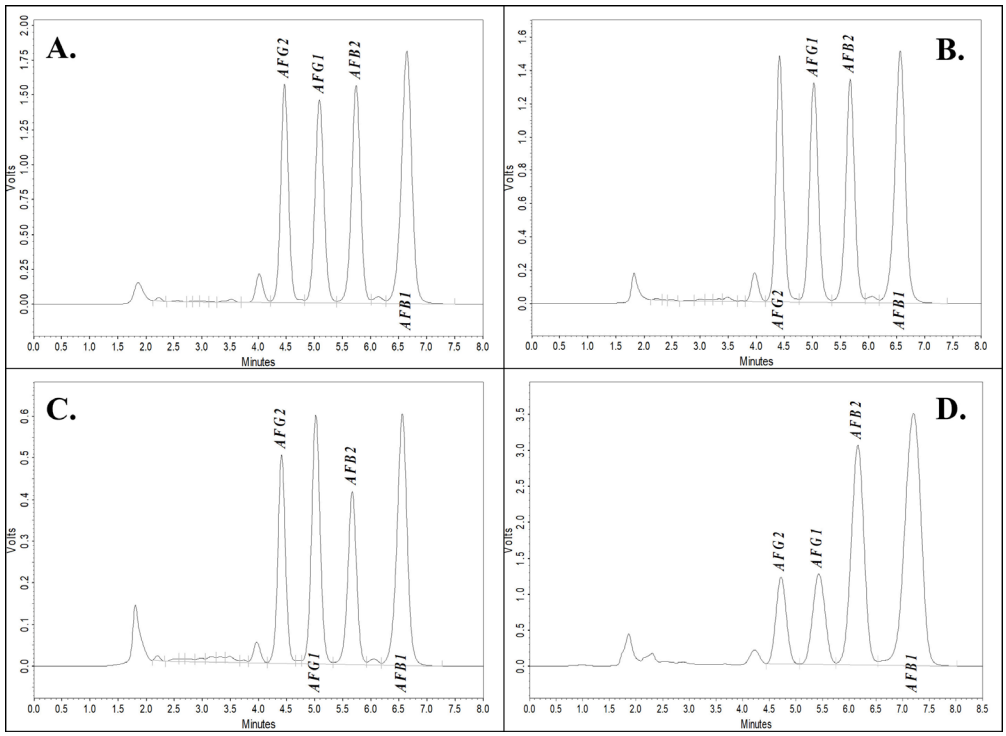


**Figure 4.** HPLC-FLD chromatograms of AFs produced by *A. parasiticus* strains SZMC 22361 (A), SZMC 22727 (B), SZMC 22728 (C) and SZMC 24773 (D) on ground corn supplemented with 10 g water during the preparation of the medium after one week of fermentation.

Based on the literature, *A. pseudotamarii* strains are known to produce only AFB1 and AFB2 (Ito et al., 2001; Ehrlich et al. 2003), which is consistent with our results. However, *A. minisclerotigenes* isolates have been reported to produce AFG1 and AFB1 mycotoxins (Pildain et al. 2008), though this was determined using diode array UV-VIS detection on a synthetic agar-based medium. Previously, Endre et al. (2019) detected all four AFs produced by *A. parasiticus*, but with a reversed ratio of AFG1 to AFB1. In our measurements, AFB1 levels were approximately twice those of AFG1, whereas in the aforementioned study, AFG1 levels were roughly double those of AFB1. This discrepancy may stem from differences in cultivation media: in the referenced study, the fungal strain was grown in a liquid medium containing malt and yeast extract, whereas in our study, a solid plant-based substrate was used.

Regarding the effect of water content on fermentation, no significant differences were observed in AF yield across all examined species and moisture conditions (Fig. 1). Consequently, for subsequent experiments, a standardized water amount of 10 g was used for cultivation. Additionally, micromorphology, biomass formation, and conidia production remained similar under the different moisture conditions (Fig. 2).

### *AF production of A. parasiticus isolates*

Due to the significantly high AF production of *A. parasiticus* strain SZMC 24773, three additional isolates from this species were included in the investigation to maximize AF yield during fermentation (Figs. 3, 4). Strains *A. parasiticus* SZMC 22727 and SZMC 22728 produced significantly lower amounts of AFB1 than the initially investigated strain SZMC 24773. However, strain SZMC 22361 exhibited a similar AFB1 production capacity (Fig. 3A).

The AFB2 production of the strains ranged from 2 μg/mg to 12 μg/mg, with only SZMC 22728 displaying a significantly lower yield than strain SZMC 24773 (Fig. 3B). Regarding AFG production, two strains (SZMC 22361 and SZMC 22727) yielded higher levels of mycotoxins than the other two *A. parasiticus* strains (Fig. 3C, D). Both AFG1 and AFG2 were present in approximately twice the amount in the extracts of SZMC 22361 and SZMC 22727 compared to SZMC 22728 and SZMC 24773.

Based on these findings, *A. parasiticus* strain SZMC 22361 was selected for further investigations, as it produces AFBs at similarly high levels as strain SZMC 24773 but exhibits the highest AFG yield among the examined strains.

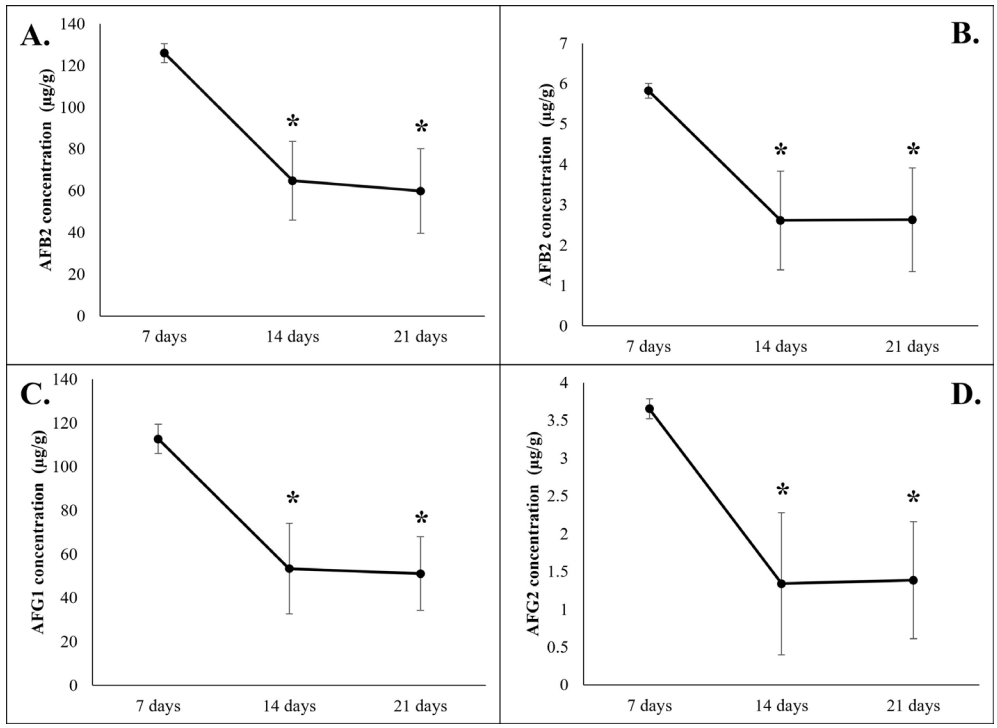


**Figure 5.** Production of AFB1 (A), AFB2 (B), AFG1 (C) and AFG2 (D) by *A. parasiticus* strain SZMC 22361 after different durations of fermentation. The asterisks show the significant differences (P ≤ 0.05) from the AF production at day 7.

### *Effect of incubation time on the AF production*

The changes in AF production by *A. parasiticus* strain SZMC 22361 were monitored over a three-week period, with AF concentrations measured on days 7, 14, and 21. The AF contents of the samples ranged from 60–126 µg/mg for AFB1, 2–6 µg/mg for AFB2, 51–113 µg/mg for AFG1, and 1–4 µg/mg for AFG2 (Fig. 5).

For all AFs, yields decreased significantly after one week of fermentation, dropping to approximately half of the original amount. Therefore, to achieve maximum toxin production with this isolate, fermentation should be terminated after one week. Similarly, in the case of an *A. flavus* strain, maximum AFB1 production was also observed after 7 days of incubation under optimal conditions (Lahouar et al. 2016).

## Conclusion

Overall, among the examined *Aspergillus* strains, *A. parasiticus* SZMC 22361 proved to be the most efficient AF producer on a ground corn substrate. The optimal cultivation conditions were determined to be a substrate-to-water ratio of 1:1 (m/m) and a fermentation period of one week.

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