

Interakcije nekih plijesni i aflatoksinogenog soja *Aspergillus flavus* NRRL 3251

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INTERACTION BETWEEN CERTAIN MOULDS AND AFLATOXIN B₁ PRODUCER *ASPERGILLUS FLAVUS* NRRL 3251

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The objective of this study was to evaluate biotic interaction between some mould species and active producer of aflatoxin B₁ *Aspergillus flavus* NRRL 3251, co-cultured in yeast-extract sucrose (YES) broth. Twenty-five mould strains of *Alternaria* spp., *Cladosporium* spp., *Mucor* spp., *A. flavus* and *A. niger*, used as biocompetitive agents, were isolated from outdoor and indoor airborne fungi, scrapings of mouldy household walls, and from stored and post-harvest maize. Aflatoxin B₁ was extracted from mould biomasses with chloroform and detected using the multitoxin TLC method. The results confirm antagonistic interaction between all strains tested. With *Alternaria* spp. and *Cladosporium* spp., aflatoxin B₁ production decreased 100 %, compared to detection in a single culture of *A. flavus* NRRL 3251 ($C_{\text{mean}} = 18.7 \mu\text{g mL}^{-1}$). In mixed cultures with *Mucor* spp., aflatoxin B₁ levels dropped to (5.6-9.3) $\mu\text{g mL}^{-1}$, and the inhibition was from 50 % to 70 %. Four of five aflatoxin non-producing strains of *A. flavus* interfered with aflatoxin production in mixed culture, and reduced AFB₁ productivity by 100 %. One strain showed a lower efficacy in inhibiting AFB₁ production (80 %) with a detectable amount of AFB₁ 3.7 $\mu\text{g mL}^{-1}$ when compared to control. A decrease in toxin production was also observed in dual cultivation with *A. niger* strains. It resulted in 100 % reduction in three strains, 90 % reduction in one strain ($C_{\text{mean}} = 1.9 \mu\text{g mL}^{-1}$) and 80 % reduction in one strain ($C_{\text{mean}} = 3.7 \mu\text{g mL}^{-1}$) inhibition.

KEY WORDS: biosynthesis, biological control, mixed cultures, mycotoxins

Aspergillus flavus, *A. parasiticus*, *A. niger* and a few other *Aspergilli* which produce aflatoxins (AFs) are closely related omnipresent microfungi that contaminate seeds and plant debris of many crops in the field during harvest, storage, and processing. Aflatoxins are highly toxic, carcinogenic, mutagenic, teratogenic and immunosuppressive compounds, and their presence in the food chain is potentially hazardous to human and animal health. Aflatoxins B₁, B₂, G₁, and G₂ are classified as Group I human carcinogens (1). Possible presence of highly toxic and carcinogenic mycotoxins in foods and foodstuffs has led to extensive research involving methods for inhibiting the synthesis of mycotoxins and of aflatoxin in particular. Mycotoxin production during storage can

be stopped by reducing mould growth, and various chemical and physical control methods have been developed to remove mycotoxins from foods and feeds (2). However, these methods are not always economical and their success varies. Therefore, there has been an increasing interest in identifying naturally occurring compounds that would limit the growth of aflatoxigenic fungi and/or their toxin production.

Another goal of research was to develop successful strategies for the use of biocontrol agents to protect crops from toxigenic fungal attack. Antifungal abilities of some beneficial microbes have been known for long, but they are only now being widely studied to used commercially. Alternatively, a number of microbes (lactic acid bacteria, *Bacillus* sp., and

saprophytic yeasts) or their enzymes were screened to select an organism suitable for detoxification of mycotoxins (3-6). The ability of several fungal cultures to prevent aflatoxin B₁ synthesis in a liquid medium has also been reported. Among these *Phoma*, *Mucor*, *Rhizopus*, *Alternaria* and *Trichoderma* species, consistently reduced aflatoxins by 90 % or more (7-10). One strategy that has greatly reduced mycotoxin contamination in grains is the biocontrol strategy which applies non-mycotoxin-producing strains (atoxicogenic strains) of the same species that competitively exclude mycotoxin producers in agricultural environments and thereby reduce AF contamination (11-13). All this inhibition may result from many factors, including competition for space and nutrients in general, competition for nutrients required for aflatoxin production, but not for growth, and production of anti-aflatoxicogenic metabolites by

co-existing microorganisms. Aflatoxin production is affected by many abiotic and biotic factors which are relatively less known.

The aim of this study was to evaluate the biotic interaction between twenty-five indigenous strains of various moulds in respect to their ability to prevent mycotoxin production by the strain *A. flavus* NRRL 3251, co-cultured in liquid medium.

MATERIALS AND METHODS

Strains

Aspergillus flavus Link ex Fres. NRRL 3251 as an active producer of aflatoxin B₁ (AFB₁), and twenty-five tested strains of *Alternaria* spp., *Cladosporium* spp., *Mucor* spp., *A. flavus* and *A. niger*, used as

Table 1 Mould cultures isolated from various sources

	Isolate number	Mould species	Source
1	8972	<i>Alternaria alternata</i>	wall scraping
2	9009	<i>A. alternata</i>	wall scraping
3	8910	<i>Alternaria</i> sp.	outdoor air
4	8658	<i>Alternaria</i> sp.	stored maize
5	8466	<i>Alternaria</i> sp.	stored maize
6	8984	<i>Cladosporium</i> sp.	wall scraping
7	8985	<i>Cladosporium</i> sp.	wall scraping
8	9052	<i>Cladosporium</i> sp.	outdoor air
9	8856	<i>Cladosporium</i> sp.	indoor air
10	8254	<i>Cladosporium</i> sp.	indoor air
11	8988	<i>Mucor</i> sp.	wall scraping
12	9002	<i>Mucor</i> sp.	wall scraping
13	8446	<i>Mucor</i> sp.	post- harvest maize
14	8449	<i>Mucor</i> sp.	post-harvest maize
15	8466	<i>Mucor</i> sp.	post-harvest maize
16	23. VII	<i>Aspergillus flavus</i>	outdoor air
17	20. VI	<i>A. flavus</i>	mountain air
18	8. VI	<i>A. flavus</i>	outdoor air
19	8. VII	<i>A. flavus</i>	outdoor air
20	17. VII	<i>A. flavus</i>	outdoor air
21	8998	<i>Aspergillus niger</i>	wall scraping
22	9021	<i>A. niger</i>	wall scraping
23	V7I	<i>A. niger</i>	outdoor air
24	V2III	<i>A. niger</i>	outdoor air
25	ATCC 16404	<i>A. niger</i>	*
	NRRL 3251	<i>A. flavus</i>	reference culture**

*America Type Culture Collection (Rockville, Maryland, USA)

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biocompetitive agents in this study, were obtained from the culture collection of our laboratory (Table 1). Some strains were naturally occurring isolates from airborne outdoor and indoor fungi, scrapings from mouldy household walls, or from stored and post-harvest maize. Cultures were identified from their macro- and microscopic morphology according to keys after subculture on Czapek, Sabouraud or Potato dextrose agar (14, 15).

In vitro aflatoxin production

Before starting with the *in vitro* interaction experiments, the reference culture *A. flavus* NRRL 3251 was characterised concerning its aflatoxin B₁ production which was carried out in duplicate on Erlenmeyer flasks containing yeast-extract sucrose (YES) medium. *A. flavus* (five strains) and *A. niger* (five strains) were previously confirmed by biosynthesis and thin layer chromatography (TLC) as non-producers of AFB₁. Cultures of *Alternaria*, *Cladosporium* and *Mucor* species were not tested for their toxigenicity. Interactive cultures (*A. flavus* NRRL 3251 and each of the tested moulds) were coinoculated in 50 mL of YES with 1 mL of each conidia suspension (of 10⁶ conidia mL⁻¹) from 7-day-old cultures grown on potato dextrose agar (PDA). Flasks were incubated for 10 days in dark at (25±2) °C, and were shaken every day. Regular biosynthesis was observed for mould growth and spore formation.

Extraction and Thin Layer Chromatography of Aflatoxin B₁

Aflatoxin B₁ was isolated and detected using multitoxin extraction and the semi-quantification TLC method according by Balzer *et al.* (16). Briefly, mixed cultures (50 mL) were homogenized with 50 mL acetonitrile:water (9:1) for ten minutes. From each filtrated sample, 50 mL was treated with *n*-hexane (2x25 mL) to remove the lipids. Saturated solution of NaHCO₃ (25 mL) and water (25 mL) were added into samples (pH=8-9) and extracted with chloroform (25 mL). Lower chloroform fraction was treated with 1 mol L⁻¹ NaOH (2x10 mL). Chloroform fraction was washed with 25 mL of water, and lower phase (containing aflatoxins) was filtered through anhydrous Na₂SO₄, evaporated to dryness, and redissolved in 0.2 mL of chloroform for TLC analysis. The detection limit for AFB₁ was 2 µg mL⁻¹ and recovery 71 %. Aflatoxin B₁ reference standard was purchased from Carol Roth (D-75) the concentration of 2 µg mL⁻¹.

Fluorescence intensities of toxin spots and standard were compared visually under UV light (366 nm). Two-dimensional TLC and spray treatment of the developed TLC plate, with 50 % sulphuric acid in ethanol were used to confirm the presence of aflatoxin B₁.

RESULTS AND DISCUSSION

Table 2 summarises the effects of all twenty-five fungal bioagents [*Alternaria alternata* (two strains), *Alternaria* spp. (three strains), *Cladosporium* spp. (five strains), *Mucor* spp. (five strains), *Aspergillus flavus* (five strains) and *A. niger* (five strains)] on the growth and production of aflatoxin B₁ by *A. flavus* NRRL 3251 after 10 days of incubation. By the final reading, all cultures showed growth, but only *A. flavus* NRRL 3251 in individual culture had 100 % mycelial coverage and complete sporulation. In general, the growth of *A. flavus* NRRL 3251 was suppressed and AFB₁ production completely inhibited when incubated with *Alternaria* or *Cladosporium* strains in mixed liquid cultures. Macroscopically, growth restriction and absent fruiting structures in co-cultures may have been caused by competition (e.g. nutritional) or by metabolites produced by these moulds which specifically inhibit aflatoxin synthesis. A pigment produced by *Cladosporium* is believed to have a compound which may be responsible for inhibiting aflatoxin B₁ production (17).

All treatments showed inhibitory effect on both the mycelial growth and aflatoxin production when compared to control. In all combinations, *Alternaria* and *Cladosporium* completely inhibited AFB₁ (100 %) in comparison with the control single culture of *A. flavus* NRRL 3251 (C_{mean} = 18.7 µg mL⁻¹). Obviously, these moulds grown as mixed cultures do not support growth, sporulation, and toxin production, and their anti-toxigenic potential is very strong. With *Mucor* spp. and *A. flavus* AFB₁ producer in mixed cultures, all five *Mucor* strains also showed a marked restrictive effect on mycelial growth of *A. flavus* in comparison with growth in the control cultures. These strains were the least antagonistic to the productive strain *A. flavus* NRRL 3251, and reduced AFB₁ levels to (5.6-9.3) µg mL⁻¹ (50 % to 70 %).

Coinoculation with ten strains of *A. flavus* and *A. niger* which were confirmed non-toxigenic showed that they were also antagonistic to *A. flavus* NRRL 3251. Among the tested aflatoxin non-producing

Table 2 Production of aflatoxin B₁ by *A. flavus* NRRL 3251 in mixed cultures with mould strains grown on yeast extract-sucrose medium (YES)

Moulds	No. of strains tested	Concentration of AFB ₁ / $\mu\text{g mL}^{-1}$	Inhibition / %
<i>A. flavus</i> NRRL3251*	1	18.7	0
<i>A. flavus</i> * <i>Alternaria</i> spp.	5	nd	100
<i>A. flavus</i> * <i>Cladosporium</i> spp.	5	nd	100
<i>A. flavus</i> * <i>Mucor</i> spp.	1	5.6	70
<i>A. flavus</i> * <i>Mucor</i> spp.	2	7.5-9.3	50-60
<i>A. flavus</i> * <i>Mucor</i> spp.	2	7.5-9.3	50-60
<i>A. flavus</i> * <i>A. flavus</i>	1	3.7	80
<i>A. flavus</i> * <i>A. flavus</i>	4	nd	100
<i>A. flavus</i> * <i>A. niger</i>	1	3.7	80
<i>A. flavus</i> * <i>A. niger</i>	1	1.9	90
<i>A. flavus</i> * <i>A. niger</i>	3	nd	100

**A. flavus* NRRL 3251, reference culture, producer of AFB₁
nd - AFB₁ is not detected
LOD=2 $\mu\text{g L}^{-1}$

strains of *A. flavus*, four out of five interfered with AFB₁ production in the culture, and reduced it 100 %. One atoxigenic strain showed lower inhibition of AFB₁ production (80 %) when compared with control. The amount of AFB₁ determined in the biomass of mixed cultures was 3.7 $\mu\text{g mL}^{-1}$ and in the control culture 18.7 $\mu\text{g mL}^{-1}$. Atoxigenic *A. flavus* strains are known to vary in enzymatic activities in the aflatoxin biosynthetic pathway (18, 19). In interactive *in vitro* studies with non-toxigenic *A. flavus* strains, Martins *et al.* (20) confirmed synergic interaction and a potential increase in aflatoxin productivity. One widespread *A. flavus* strain showed a remarkable synergic activity with a competitive strain, resulting in a 106.5 % increase in respect to control average (*A. parasiticus*).

A decrease in toxin production was also observed in dual cultivation with *Aspergillus niger*. The three strains were aggressive inhibitors of *A. flavus* growth, and macroscopic observations of mixed cultures showed highly predominant sporulating of *A. niger*,

whereas AFB₁ was not detected. One strain led to a 90 % inhibition ($C_{\text{mean}} = 1.9 \mu\text{g mL}^{-1}$) and another in 80 % inhibition ($C_{\text{mean}} = 3.7 \mu\text{g mL}^{-1}$), primarily due to the action of gluconic acid, which is produced by *A. niger* (21-23).

CONCLUSION

In our study, a number of filamentous fungi of four genera, *Alternaria*, *Cladosporium*, *Mucor* and *Aspergillus*, showed to be antagonistic to the reference toxigenic strain of *A. flavus* NRRL 3251, and had the potential to inhibit its AFB₁ production. Of 25 mould strains assayed, 68 % reduced AFB₁ production of the highly toxigenic *A. flavus* by 100 % while 32 % showed AFB₁ inhibition from 90 % to 50 %. Aflatoxin B₁ production in all mixed cultures was lower (between 1.9 $\mu\text{g mL}^{-1}$ to 9.3 $\mu\text{g mL}^{-1}$) than in control cultures (*A. flavus* NRRL 3251, $C_{\text{max}} = 18.7 \mu\text{g mL}^{-1}$).

It is evident that certain moulds isolated from the environment can effectively control the mycelial growth of aflatoxigenic strains, and may be considered effective AFB₁ inhibitors, as they are able to reduce AFB₁ production from 50 % to 100 %. These mould strains may prove useful in limiting or preventing toxigenesis and in removing toxigenic strains.

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SažetakINTERAKCIJE NEKIH PLJESNI I AFLATOKSINOGENOG SOJA *Aspergillus flavus* NRRL 3251

Cilj rada bio je procijeniti biotske interakcije između sojeva različitih vrsta plijesni i kontrolnog soja *Aspergillus flavus* NRRL 3251, producenta aflatoksina B₁ (AFB₁).

Inhibitorno djelovanje u miješanim kulturama na tvorbu AFB₁ ispitano je na dvadeset pet sojeva *Alternaria*, *Cladosporium*, *Mucor* i *Aspergillus* vrsta izoliranih iz zraka, strugotina pljesnivih zidova te uskladištenog i prezimljenog kukuruza. Biosinteze su provedene u tekućoj hranjivoj podlozi s kvaščevim ekstraktom (YES-bujon). Ekstrakcije AFB₁ iz biomase izvršene su multitoksinskom metodom tankoslojne kromatografije. Rezultati biotskih interakcija pokazali su antagonistički odnos svih testiranih sojeva. *Alternaria* i *Cladosporium* vrste simultano inokulirane sporama *A. flavus* NRRL 3251 inhibirale su tvorbu AFB₁ 100 % u odnosu na dokazani toksin u kontrolnoj biosintezi (konc. 18,7 µg mL⁻¹). U miješanim kulturama vrstama roda *Mucor* dokazane su padajuće koncentracije AFB₁ (9,3 µg mL⁻¹, 7,5 µg mL⁻¹ i 5,6 µg mL⁻¹), odnosno inhibicija tvorbe toksina 50 % do 70 %. Atoksinogeni sojevi *A. flavus* inhibirali su tvorbu AFB₁ 80 % (1 soj, konc. 3,7 µg mL⁻¹) i 100 % (4 soja). Antagonističko djelovanje prema toksinogenom soju, smanjujući tvorbu AFB₁ u rasponu 80 % do 100 % (konc. 1,9 µg mL⁻¹ i 3,7 µg mL⁻¹), dokazano je u uzgojnim biosintezama s *A. niger*.

KLJUČNE RIJEČI: biosinteza, biološka kontrola, miješane kulture, mikotoksini

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