Interakcije nekih plijesni i aflatoksinogenog soja Asspergillus flavus NRRL 3251

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Short Communication

INTERACTION BETWEEN CERTAIN MOULDS AND AFLATOXIN B_1 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 B_1 *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 B_1 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 B_1 production decreased 100 %, compared to detection in a single culture of *A. flavus* NRRL 3251 (C_{mean}=18.7 μg mL^{-1}). In mixed cultures with *Mucor* spp., aflatoxin B_1 levels dropped to (5.6-9.3) μg 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 AFB_1 productivity by 100 %. One strain showed a lower efficacy in inhibiting AFB_1 production (80 %) with a detectable amount of AFB_1 3.7 μg 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_{mean}=1.9 μg mL^{-1}) and 80 % reduction in one strain (C_{mean}=3.7 μg mL^{-1}) inhibition.

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

Aspergillus flavus, A. parasiticus, *A. nominus* 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 B_1, B_2, G_1, and G_2 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 B_1 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 (atoxigenic 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-aflatoxigenic 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 B_1 (AFB_1), 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	Alternaria alternata	wall scraping
2	9009	A. alternata	wall scraping
3	8910	Alternaria sp.	outdoor air
4	8658	Alternaria sp.	stored maize
5	8466	Alternaria sp.	stored maize
6	8984	Cladosporium sp.	wall scraping
7	8985	Cladosporium sp.	wall scraping
8	9052	Cladosporium sp.	outdoor air
9	8856	Cladosporium sp.	indoor air
10	8254	Cladosporium sp.	indoor air
11	8988	Mucor sp.	wall scraping
12	9002	Mucor sp.	wall scraping
13	8446	Mucor sp.	post- harvest maize
14	8449	Mucor sp.	post-harvest maize
15	8466	Mucor sp.	post-harvest maize
16	23. VII	Aspergillus flavus	outdoor air
17	20. VI	A. flavus	mountain air
18	8. VI	A. flavus	outdoor air
19	8. VII	A. flavus	outdoor air
20	17. VII	A. flavus	outdoor air
21	8998	Aspergillus niger	wall scraping
22	9021	A. niger	wall scraping
23	V7I	A. niger	outdoor air
24	V2III	A. niger	outdoor air
25	ATCC 16404	A. niger	*
	NRRL 3251	A. flavus	reference culture**

*America Type Culture Collection (Rockville, Maryland, USA)
**Northern Regional Research Laboratory, Peoria

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 B_1 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 AFB_1. 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 10^6 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 B_1

Aflatoxin B_1 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 $NaHCO_3$ (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 Na_2SO_4, evaporated to dryness, and redissolved in 0.2 mL of chloroform for TLC analysis. The detection limit for AFB_1 was 2 μg mL^{-1} and recovery 71 %. Aflatoxin B_1 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 B_1.

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 B_1 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 AFB_1 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 B_1 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 AFB_1 (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* AFB_1 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 AFB_1 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 B_1 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 / $\mu g\ mL^{-1}$	Inhibition / %
A. flavus NRRL3251*	1	18.7	0
*A. flavus** *Alternaria*spp.	5	nd	100
*A. flavus** *Cladosporium*spp.	5	nd	100
*A. flavus** *Mucor*spp.	1	5.6	70
*A. flavus** *Mucor*spp.	2	7.5-9.3	50-60
*A. flavus** *Mucor*spp.	2	7.5-9.3	50-60
*A. flavus** *A. flavus*	1	3.7	80
*A. flavus** *A. flavus*	4	nd	100
*A. flavus** *A. niger*	1	3.7	80
*A. flavus** *A. niger*	1	1.9	90
*A. flavus** *A. niger*	3	nd	100

**A. flavus* NRRL 3251, reference culture, producer of AFB_1
nd - AFB_1 is not detected
LOD=2 $\mu g\ L^{-1}$

strains of *A. flavus*, four out of five interfered with AFB_1 production in the culture, and reduced it 100 %. One atoxigenic strain showed lower inhibition of AFB_1 production (80 %) when compared with control. The amount of AFB_1 determined in the biomass of mixed cultures was 3.7 $\mu g\ mL^{-1}$ and in the control culture 18.7 $\mu g\ mL^{-1}$. 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 AFB_1 was not detected. One strain led to a 90 % inhibition (C_{mean}=1.9 $\mu g\ mL^{-1}$) and another in 80 % inhibition (C_{mean}=3.7 $\mu g\ mL^{-1}$), 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 AFB_1 production. Of 25 mould strains assayed, 68 % reduced AFB_1 production of the highly toxigenic *A. flavus* by 100 % while 32 % showed AFB_1 inhibition from 90 % to 50 %. Aflatoxin B_1 production in all mixed cultures was lower (between 1.9 $\mu g\ mL^{-1}$ to 9.3 $\mu g\ mL^{-1}$) than in control cultures (*A. flavus* NRRL 3251, C_{max}=18.7 $\mu g\ mL^{-1}$).

It is evident that certain moulds isolated from the environment can effectively control the mycelial growth of aflatoxigenic strains, and may be considered effective AFB_1 inhibitors, as they are able to reduca AFB_1 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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Sažetak

INTERAKCIJE NEKIH PLIJESNI 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 B_1 (AFB_1).
Inhibitorno djelovanje u miješanim kulturama na tvorbu AFB_1 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 AFB_1 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 AFB_1 100 % u odnosu na dokazani toksin u kontrolnoj biosintezi (konc. 18,7 μg mL^{-1}). U miješanim kulturama vrstama roda *Mucor* dokazane su padajuće koncentracije AFB_1 (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 AFB_1 80 % (1 soj, konc. 3,7 μg mL^{-1}) i 100 % (4 soja). Antagonističko djelovanje prema toksinogenom soju, smanjujući tvorbu AFB_1 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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