Effect of *Rhizopus* and *Neurospora* spp. on growth of *Aspergillus flavus* and *A. parasiticus* and accumulation of aflatoxin B_1 in groundnut

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The influence of *Rhizopus* and *Neurospora* spp. on growth and aflatoxin B_1 accumulation of *Aspergillus flavus* and *A. parasiticus* was investigated. When inoculated simultaneously with *Rhizopus* or *Neurospora* spp. on mashed groundnuts, *Aspergillus* spp. were able to grow, but to a lesser extent and with visually different mycelial development and sporulation behaviour. Accumulation of aflatoxin B_1 observed during a 6 wk incubation, averaged 34% in mixed cultures with *Rhizopus* spp. and 1·7% in mixtures with *Neurospora* spp., as compared to pure cultures of *Aspergillus* spp. On aqueous groundnut extract, selected strains of *Rhizopus* and *Neurospora* showed the ability to cause an exhaustion particularly of C compounds, inhibiting *Aspergillus* growth and aflatoxin B_1 accumulation. There was also evidence for the formation of *Rhizopus* and *Neurospora* metabolites inhibiting growth and/or aflatoxin B_1 production. In addition *Rhizopus* and *Neurospora* strains degraded aflatoxin B_1.

Key words: *Aspergillus, Rhizopus, Neurospora,* Mixed culture, Aflatoxins.

Under natural conditions, the growth of *Aspergillus flavus* Link: Fr. var. *flavus* and *A. parasiticus* Speare as well as the accumulation of aflatoxins are influenced by a number of factors. These include the genetic make-up of these species determining their capability to produce and degrade aflatoxins (Hesseltine, 1979); physical environment, i.e. temperature (Wieman *et al.*, 1986; Magan & Lacey, 1984; Bullerman, 1985), water activity (Wieman *et al.*, 1986; Magan & Lacey, 1984), pH (Bullerman, 1985), aeration (Hesseltine, 1979); chemical composition of the substrate, i.e. presence of growth inhibitors (Zaika & Buchanan, 1987) or precursors of aflatoxin biosynthesis (Buchanan & Stahl, 1984); and the influence of other micro-organisms. With regard to the latter, the influence of microflora interactions in grain storage situations has been reported (Magan & Lacey, 1985; Horn & Wicklow, 1983; Ehrlich *et al.*, 1985). Another situation where microflora interaction plays a role is in the fermentation of foods. In this respect, Ko (1974) reported that *Rhizopus microsporus* var. *oligosporus* (Saito) Schipper & Stalpers NRRL 5905 and *Neurospora sitophila* Shear & B. O. Dodge ATCC 14151 opposed to the accumulation of aflatoxins by *Aspergillus flavus* CBS 120.62 during the fermentative production of tempe and oncom, respectively. He referred to this as 'self-protection of fermented foods'.

The aim of this paper is to broaden the data reported by Ko and to investigate the nature of the self-protection. Hypothetically, several processes may prevent or reduce the accumulation of aflatoxins in fermented foods. These could be classified as follows: (a) competition for nutrients or aflatoxin precursors; (b) formation of substances inhibitory to the biosynthesis of aflatoxins; and (c) biodegradation of aflatoxins which were formed previously. This paper attempts to evaluate the role of such effects.

MATERIALS AND METHODS

Cultures and biological materials

The following cultures were obtained from collections: *Aspergillus flavus* 006 (NRRL 5906 = CBS 120.62), *A. parasiticus* 013 (NRRL 2999) and 014 (ATCC 15517 = CBS 260.67), *Rhizopus oligosporus* 571 (NRRL 5865) and 575 (NRRL 5905), *R. microsporus* van Tiegh. var. *microsporus* 574 (CBS 699.68), *Neurospora crassa* Shear & B. O. Dodge 421 (ATCC 24914) and 425 (ATCC 14151). Pure cultures isolated from Indonesian tempe included: *R. oryzae* Went & Prinsen Geerligs 581, *R. oligosporus* 592, 593, 594, T-8, T-9 and T-10. The strains *R. microsporus* var. *chinensis* (Saito) Schipper & Stalpers 589 and *R. oligosporus* 591 were isolated from Indonesian tempe inoculum, whereas *R. microsporus* 573, *R. oryzae* 583 and 588 were isolated from Dutch tempe. The following cultures were isolated from Indonesian oncom: *R. oligosporus* 0-10 and 0-13, and *Neurospora* sp. 428, 429, 430, 0-3, 0-14, 0-16, 0-17, 0-18 and 0-19.

Groundnuts were redskins obtained from a Dutch feed mill.

Cultivation and maintenance of fungi

Aspergilli were grown at 28–30 °C on slopes of Malt Extract Agar (MEA, Oxoid CM59) and were maintained at 4° on the

same medium. *Rhizopus* and *Neurospora* cultures were grown at 28–30° on slopes of Soya Malt Extract Agar (SMEA) obtained as follows: 150 g dehulled soya-beans were soaked in 250 ml tapwater for 24 h at room temperature; after discarding the soak water, the beans were boiled 30 min in fresh tapwater and ground to a mash with an equal weight of fresh tapwater using a Waring blender. After addition of 50 g MEA the volume was made up to 1 l and the resulting medium was sterilized for 20 min at 121°. The cultures were maintained at 4° on the same medium.

Preparation of spore suspensions

Cultures were brought to intensive sporulation by incubation for 7 d at 30° on slopes of taugé agar (TA) prepared as follows: 100 g taugé (Mung bean sprouts) were boiled 30 min in 750 ml tapwater, followed by filtration through filterpaper. After addition of 60 g sucrose and 20 g agar, the volume was made up to 1 l, the agar was dissolved by boiling and the medium was sterilized for 15 min at 121°. Fungal spores were harvested by adding 10 ml sterile 0·02% Tween 80 in distilled water and dislodging by vibration on a whirl-mixer. The suspension was centrifuged 30 min at 3000 rev. min^{-1} in sterile centrifuge tubes, washed twice with portions of 10 ml sterile distilled water and suspended in 10 ml sterile distilled water. Viable spores were counted in pour plates of Rose Bengal Chlortetracycline Chloramphenicol agar (RBCC, Difco Cook 0703-01 with addition of filter sterilized solutions containing 100 mg chloramphenicol and 100 mg chlortetracycline per l medium) incubated 48 h at 30°. Spore suspensions were kept at −20°.

Preparation of groundnut extract

Groundnut extract (GE) was obtained by shredding 1 kg raw groundnuts (Moulinex, France) followed by grinding with 2 l tapwater in a Waring blender. After straining, the residue was ground again with 1 l tapwater and strained. The two filtrates were combined, heated for 15 min in an autoclave at 121° and centrifuged 15 min at 1500 *g* ($r_{av.}$ 30 cm). The supernatant was again heated and centrifuged as before to remove heat-labile components. The supernatant was lyophilized after filtration in a Büchner funnel through paper (Schleicher & Schüll 520-b). The lyophilized material was dissolved in demineralized water and kept at −20° as a concentrated solution (50% w/w dry matter content). Before use, the required quantity of concentrate was diluted 50 times to GE of 1% w/w dry matter content and tyndallized by steaming 30 min on 3 consecutive days.

Preparation of groundnut extract agar

Groundnut Extract Agar (GEA) was prepared by dissolving 2% agar (BBL 11849, Becton Dickinson & Co., U.S.A.) in GE and sterilizing 15 min at 121°.

Preparation of shredded groundnut substrate

Shredded Groundnut Substrate (SGS) was prepared by shredding raw groundnuts (Moulinex, France) followed by grinding 2 parts by weight of shredded groundnut with 1 part of tapwater in a Waring blender. The resulting mash was sterilized for 30 min at 121° in 5 cm thick layers in crystallizing glass dishes. The resulting hard mass was broken up and distributed in 50 g portions in sterile Petri dishes under aseptic conditions.

Inoculation and incubation of experimental substrates

Experiments on GE were carried out with 25 ml portions of liquid covering the bottom of 250 ml glass jars, with loosely fitted 'twist-off' lids. Inoculation was with 0·1 ml of spore suspension containing 10^5 viable spores ml^{-1} for each strain. Incubation was at 30° for 7 d.

Experiments on GEA were carried out in glass Petri dishes (11 cm diam). After depositing a sterile filter paper disc (0·5 cm diam) on the centre of the agar, an inoculum of 0·25 ml spore suspension of each tested strain containing 10^5 viable spores ml^{-1} was pipetted onto the disc. Incubation was at 30° for periods up to 7 d.

Experiments on SGS were carried out in plastic Petri dishes (14 cm diam, Art. 639102, Greiner, The Netherlands). The inoculum consisted of 0·1 ml spore suspension containing 10^5 viable spores ml^{-1} for each strain tested, on a centrally placed filter paper disc as with GEA. Incubation was at 30° for 6 wk. During this period, Petri dishes were wrapped in polyethylene to prevent excessive moisture losses.

The water activity (a_w) of SGS was 0·98 at inoculation; at the end of the experiments the a_w was 0·94–0·97.

Determination of growth (biomass)

GEA slabs with fungal mass were removed from their respective Petri dishes and transferred quantitatively into a 600 ml glass beaker containing 100–150 ml distilled water. After covering the beaker with a glass lid, the water was quickly brought to boiling using a microwave oven (Philips 2010G), resulting in a separation of the fungal mass and the medium which dissolved in the water. After cooling to approx. 70°, the suspension was filtered onto a pre-weighed filter paper (Schleicher & Schüll 520-b) in a Büchner funnel and the residue was washed with 100 ml hot distilled water to remove adhering medium as much as possible. The filter + residue were dried to constant weight in approx. 20 h at 80°. The biomass determination was corrected for residual medium absorbed in the filter by filtering sterile GEA in the same way. No correction could be made for traces of medium trapped in mycelium.

Visual inspection of mixed cultures

At regular intervals the exterior of the mixed cultures was observed and compared with pure cultures of the same age on the same substrate. Colony diameter and shape, mycelium height, extent and colour of sporulation were the major criteria for the evaluation of gradual shifts taking place in the balance between the strains growing together. On SGS it took 5–6 wk until a 'stable' exterior was reached.

Estimation of aflatoxin B_1 content

A simplified extraction without clean-up was used as follows: the sample (5 ml GE culture or 10 g crushed SGS culture) was extracted with respectively 10 or 20 ml chloroform in a weighed 50 ml wide-neck polyethylene vial for 2 h at room temperature by shaking at 150 rev. min^{-1} on an orbital shaker (Gallenkamp). Corrections for evaporation were made by weighing. 1–10 µl chloroform extracts were directly spotted (Drummond capillary pipettes) on HPTLC plates (Merck, Darmstadt, Art. 5631) which had been activated at 103–105° for 2 h. On each plate, appropriate amounts of quantitative aflatoxin B_1 standards containing 1 µg ml^{-1} and 10 µg ml^{-1}, and of a qualitative standard containing aflatoxins B_1, B_2, G_1 and G_2 were run simultaneously. The plates were developed twice in the same direction: first with diethylether, and subsequently with chloroform:acetone (90:10 v/v). Quantitation was carried out with a TLC-densitometer (Shimadzu CS-910 with Xenon lamp). Using this method, the detection level was at 10 p.p.b. aflatoxin B_1.

Competition experiments

Competition experiments consisted of two stages. During the first stage, growth of a *Rhizopus* or *Neurospora* pure culture was established for 7 d as described under 'inoculation and incubation of experimental substrates'. For the second stage, the culture liquid was subsequently separated by filtration (Schleicher & Schüll 520-b), supplemented as specified below, sterilized 15 min at 121°, and inoculated and incubated with an *Aspergillus* pure culture as described above. Supplements added during the second stage were: nil (control: exhausted medium); concentrated GE to reconstruct the original concentration of 1% GE dry matter content; 5% w/v mixed carbon source consisting of 2·5% w/v glucose (Art. 8342, Merck, FRG) and 2·5% w/v sucrose (Art. 7651, Merck, FRG); or 0·5% mixed nitrogen source consisting of 0·34% w/v L-asparagine (No. 37020, BDH Biochemicals, Poole, England) and 0·16% w/v NH_4NO_3 (Art. 1188, Merck, FRG).

Degradation of aflatoxin B_1

Experiments were carried out on aflatoxin B_1 produced by *Aspergillus* pure culture in GE, and on pure aflatoxin B_1 added to GE. After supplementation as specified below, and sterilization for 15 min at 121°, the substrates were inoculated with *Rhizopus* or *Neurospora* pure cultures and incubated as described above. Some experimental treatments were supplemented with concentrated GE as described under 'competition experiments'.

RESULTS

Growth on groundnut extract agar

Table 1 represents the growth of pure and simultaneously inoculated mixed cultures of *A. flavus* 006, *A. parasiticus* 014, *R. oligosporus* 575 and *N. crassa* 421. Mixed cultures with *R. oligosporus* showed a biomass production and exterior similar to pure *R. oligosporus*. However, the mixed culture with *A. flavus* showed more vertical mycelium development after 4 d of incubation. Mixed cultures with *N. crassa* showed signs of inhibition. The exterior resembled that of the pure *N. crassa* culture but both biomass production and sporulation were retarded. After 5 d of incubation, the mixed culture with *A. parasiticus* showed *Aspergillus* mycelium development and sporulation at the edges of the colony.

Growth and aflatoxin B_1 accumulation in shredded groundnut substrate

During the 6 wk incubation period, mixed cultures of *Aspergillus* and *Rhizopus* strains showed a variation of exterior changes. In most cases, *Rhizopus* mycelium showed clearly visible growth, and was mixed with, or even overgrown by, fluffy *Aspergillus* mycelium. Table 2 summarizes exterior features of the mixed cultures; in mixed cultures with *Aspergillus*, sporulation varied in extent and colour. *Neurospora/Aspergillus* mixed cultures showed a more constant behaviour. They were all gradually dominated by fluffy *Aspergillus* mycelium but sporulation remained absent in all tested mixtures. Table 2 also presents the aflatoxin B_1 accumulated in SGS cultures after 6 wk incubation. In *Rhizopus/Aspergillus* mixtures, accumulated aflatoxin B_1 was 0–179% of that in pure *Aspergillus* cultures, with an average of 34% (S.D. 41%). Although most *Rhizopus* mixed cultures with low aflatoxin B_1 content also showed reduced or no

Table 1. Growth (as mg dry biomass) and exterior of pure and mixed cultures of *Aspergillus flavus*, *A. parasiticus*, *Rhizopus oligosporus*, and *Neurospora crassa*, inoculated simultaneously on groundnut extract agar and incubated 6 d at 30°

Incubation (d)	2	3	4	5	6
A. flavus 006	834(S)[1]	1265S[2]	1196S	1206S	958S
A. parasiticus 014	653(S)	1211S	1235S	1254S	930S
R. oligosporus 575	673(S)	662S	538S	570S	524S
N. crassa 421	834(S)	957(S)	924S	918S	652S
A. flavus 006 + *R. oligosporus* 575	733R[3]	712R	618RV[4]	748RV	696RV
A. flavus 006 + *N. crassa* 421	644N[5]	657N(S)[6]	674N(S)	701N(S)	837N(S)
A. parasiticus 014 + *R. oligosporus* 575	705R	752R	702R	667R	673R
A. parasiticus 014 + *N. crassa* 421	660N	709N(S)	725N(S)	894NAp[7]	722NAp

[1] (S): start of sporulation; [2] S: fully sporulated; [3] R: exterior identical to *Rhizopus* pure culture; [4] RV: similar to *Rhizopus* pure culture but more vertical mycelium development; [5] N: exterior identical to *Neurospora* pure culture; [6] N(S): similar to *Neurospora* pure culture but with reduced sporulation; [7] NAp: mainly *Neurospora* with development of sporulated *A. parasiticus* at the edges of the mixed colony.

Table 2. Aflatoxin B_1 accumulated by mixed cultures of *Aspergillus flavus* 006, *A. parasiticus* 013 and *A. parasiticus* 014 with *Rhizopus* or *Neurospora* strains grown on shredded groundnut substrate for 6 wk at 30°

	A 006[1]		A 013		A 014	
Aspergillus culture	aflatoxin B_1	exterior[2]	aflatoxin B_1	exterior	aflatoxin B_1	exterior
Mixed culture with:						
None (control)	71 p.p.m. = 100%		117 p.p.m. = 100%		17 p.p.m. = 100%	
R 571[1]	55[3]	FSl	43	FSd	53	Sd
R 573	2	FSd	52	FSSd	65	FSl
R 574	4	Sl	68	FSSd	76	Sd
R 575	131	FSSd	163	FSSd	33	Sl
R 581	≪[4]		≪		≪	
R 583	113	FSd	2	F	11	F
R 588	4	F	71	FSl	4	F
R 589	179		31		49	
R 591	5	F	11	F	0·4	F
R 592 (T-4)	8	Sd	≪	FSl	44	Sl
R 593 (T-6)	2		6		0·2	
R 594 (T-7)	6	Sd	21	SSd	2	FSl
R T-8	90	Sd	28	SSl	22	Sd
R T-9	9	FSSd	49	SSd	19	Sd
R T-10	13	Sl	58	FSl	14	Sd
R 0-10	11	Sd	48	FSl	7	FSl
R 0-13	34	FSl	58	FSl	55	FSd
N 421[1]	1	F	1	F	6	F
N 425	2	F	20	F	0·2	F
N 428 (0-4)	≪	F	2	F	≪	F
N 429 (0-7)	≪	F	≪	F	≪	F
N 430 (0-15)	0·2	F	4	F	≪	F
N 0-3	≪	F	2	F	0·3	F
N 0-14	0·2	F	2	F	0·5	F
N 0-16	0·2	F	3	F	0·7	F
N 0-17	1	F	3	F	0·6	F
N 0-18	0·2	F	3	F	0·2	F
N 0-19	1	F	9	F	0·3	F

[1] A: *Aspergillus*; R: *Rhizopus*; N: *Neurospora*.

[2] F: fluffy (fan-shaped) *Aspergillus* mycelium present; S: some *Aspergillus* sporulation in patches; SS: extensive *Aspergillus* sporulation; l: *Aspergillus* sporulation in light colour shades (yellow, green); d: *Aspergillus* sporulation in dark colour shades (brown, black-brown).

[3] Expressed as % of corresponding control culture.

[4] ≪: not detectable.

Aspergillus sporulation, there was no consistent relationship between extent or colour of *Aspergillus* sporulation and aflatoxin B_1 content. There were non-sporulated mixed cultures with low (R 593) as well as high (R 589) aflatoxin content. Likewise, heavily sporulated mixed cultures could have high (R 573) as well as low (R 594) aflatoxin contents. In contrast, *Neurospora*/*Aspergillus* mixed cultures showed a consistent absence of *Aspergillus* sporulation and a strong reduction of accumulated aflatoxin B_1 concentrations with an average of 1·7% (S.D. 3·6%).

Competition and inhibition in groundnut extract

For the investigation of interactive effects, combinations were chosen which resulted in maximum inhibition of *Aspergillus* sporulation and aflatoxin B_1 accumulation in SGS, i.e. *Aspergillus* 013 with *Rhizopus* 581 or *Neurospora* 429. Table 3 shows the biomass of *Aspergillus* 013 and its aflatoxin B_1 accumulation after 7 d in GE. If the same *Aspergillus* strain was inoculated on GE on which *Rhizopus* 581 or *Neurospora* 429 had previously been grown for 7 d, the biomass production was strongly reduced and no aflatoxin B_1 could be detected. Reconstitution of GE components limiting the growth in GE exhausted by previous *Rhizopus* or *Neurospora* cultivation was carried out by adding concentrated GE to its original concentration. On such reconstituted media, *Aspergillus* biomass production was still considerably less than in the control, whereas aflatoxin B_1 could not be detected. The enrichment of exhausted GE with 5% mixed carbon source resulted in higher *Aspergillus* biomass production than in the control, but much less than would be calculated on the basis of available carbon. The aflatoxin B_1 accumulation was even less than in the control. The enrichment of exhausted GE with 0·5% mixed nitrogen source had no significant effect on biomass or aflatoxin B_1 production.

Degradation of aflatoxin B_1 in groundnut extract

Table 4 shows a representation of experiments carried out with aflatoxin B_1 produced *in situ* as well as with pure aflatoxin B_1 added to GE. Aflatoxin B_1 produced by *Aspergillus* 013 in GE was quite stable during the 3 wk duration of the experiment after the culture filtrate had been sterilized by autoclaving (controls with and without GE reconstitution were included). Although *Rhizopus* 581 and *Neurospora* 429 could hardly grow on GE exhausted by *Aspergillus* 013, both strains showed the ability to reduce the aflatoxin B_1 content of the medium. This was accompanied by the formation of unidentified fluorescent components with higher R_f values compared to aflatoxin B_1. If GE exhausted by *Aspergillus* 013 was reconstituted with concentrated GE, *Rhizopus* 581 biomass production was enhanced compared to its control whereas *Neurospora* 429 growth was similar to its control. Under these circumstances, the ability of both strains to degrade aflatoxin B_1 was similar as under conditions of poor growth. In GE to which aflatoxin B_1 had been added, *Rhizopus* 581 growth was slightly inhibited, whereas that of *Neurospora* 429 was not affected. Both strains degraded aflatoxin B_1 with the formation of multiple unidentified fluorescent compounds with higher R_f values than aflatoxin B_1.

Table 3. Growth (mg biomass dry matter) and aflatoxin B_1 accumulation (p.p.m.) by *A. parasiticus* 013 in groundnut extract after previous growth of *Rhizopus* or *Neurospora* pure cultures

Aspergillus parasiticus 013	Growth (mg)	aflatoxin B_1 (p.p.m.)
Previous culture on GE:		
None (A 013[1] control)	52	1·05
R 581[1] → A 013	16	≪[5]
R 581 → A 013 + GE[2]	34	≪
R 581 → A 013 + C[3]	115	0·47
R 581 → A 013 + N[4]	5	≪
N 429[1] → A 013	< 1	≪
N 429 → A 013 + GE	29	≪
N 429 → A 013 + C	73	0·45
N 429 → A 013 + N	7	≪

[1] A: *Aspergillus*; R: *Rhizopus*; N: *Neurospora*; [2] +GE: concentrated GE added to give 1% w/v GE dry matter; [3] +C: 5% w/v mixed carbon source added; [4] +N: 0·5% w/v mixed nitrogen source added; [5] ≪: not detectable.

DISCUSSION

In this investigation, the interaction of mixed cultures was examined under conditions of simultaneous inoculation, since this is the most likely situation during the fermentation of substrates using sub-standard (contaminated) starters. From the results on GEA we concluded that it may take considerable time before an equilibrium between the components of a mixed fungal culture is reached. Consequently, the experiments on SGS were extended to a 6 wk period. On SGS, the behaviour of *Rhizopus* towards *Aspergillus* strains and *vice versa* was variable. *Aspergillus* sporulation was observed in 47% of mixed *Rhizopus* cultures with low, i.e. < 10% of control, aflatoxin content. Of mixed cultures with higher aflatoxin contents, 82% showed *Aspergillus* sporulation. No relationship between extent or colour of sporulation or nature of mycelium development, e.g. smooth, fluffy, homogenous or patchy, and aflatoxin B_1 accumulation was observed. Remarkably, *Aspergillus* sporulation was absent in all mixed cultures with *Neurospora*, and the mycelium development was very much alike. Nevertheless, this was not due to similarity between the *Neurospora* strains, considering the variation in their growth and sporulation in pure culture. Our data confirm the behaviour of *Neurospora* 425 (ATCC 14151) on groundnut medium reported earlier by Ko (1974) after a 17 d incubation period. On the other hand, Ko (1974) reported approx. 50%

Table 4. Biodegradation of aflatoxin B_1 in groundnut extract at 30° by *Rhizopus* 581 and *Neurospora* 429

	0	1		2		3	
Incubation period (wk)	AF-B_1[1] (p.p.m.)	AF-B_1 (p.p.m.)	Biomass (mg)	AF-B_1 (p.p.m.)	Biomass (mg)	AF-B_1 (p.p.m.)	Biomass (mg)
A 013[2] → - (control)	0·41[3]	0·39	5	0·42	5	0·32	4
A 013 → - + GE[5] (control)	0·41	0·50	21	0·48	16	0·50	19
GE → R 581[2]	—[10]	—	48	—	41	N.D.[11]	N.D.
A 013 → R 581	0·41	0·20[6]	10	0·22[7]	7	0·11[8]	8
A 013 → R 581 + GE	0·41	0·17[6]	73	0·17[7]	63	0·16[8]	65
GE → N 429[2]	—	—	66	—	59	N.D.	N.D.
A 013 → N 429	0·41	0·09[6]	10	0·03[7]	8	0·01[8]	7
A 013 → N 429 + GE	0·41	0·23[6]	60	0·04[7]	47	0·01[8]	48
GE + AF-B_1 (control)	0·89[4]	0·89	4	0·90	4	0·89	4
GE + AF-B_1 → R 581	0·89	0·55	36	0·24	39	0·29[9]	25
GE + AF-B_1 → N 429	0·89	0·45	51	0·37	53	0·14[9]	49

[1] AF-B_1: Aflatoxin B_1; [2] A: *Aspergillus*; R: *Rhizopus*; N: *Neurospora*; [3] Aflatoxin B_1 produced *in situ* by A 013; [4] pure aflatoxin B_1 added to GE; [5] +GE: addition of concentrated GE to 1% w/v GE dry matter; [6] single spot of fluorescent metabolite with R_f > aflatoxin B_1 [7] single spot of fluorescent metabolite with R_f > spot[6]; [8] single spot of fluorescent metabolite with R_f > spot[7]; [9] multiple fluorescent spots with R_f > aflatoxin B_1; [10] —: not detectable; [11] N.D.: not determined.

reduced aflatoxin B_1 content in tempe (fermented soya-beans) inoculated with *Rhizopus* 575 (NRRL 5905) and *Aspergillus* 006 (CBS 120.62). Our data indicate that the ability of *Rhizopus* 575 to oppose aflatoxin B_1 accumulation on groundnut was observed only with *Aspergillus* 014. Considering the high water activity of the media used, the availability of water is not likely to be responsible for the observed variation. However, this different behaviour might be explained by the different substrate (soya *versus* groundnut, respectively) and incubation periods (17 d *versus* 6 wk, respectively) employed. However, we observed that the majority of the *Rhizopus* mixed cultures caused reduced aflatoxin B_1 accumulation in groundnut. The variations in exterior and aflatoxin B_1 content in mixed cultures with *Rhizopus* underlines the involvement of a complex of factors influencing aflatoxin B_1 accumulation. Some of these factors were investigated in mixed cultures which showed good mycelium growth by *Aspergillus* but where aflatoxin B_1 and sporulation remained absent.

Simultaneously inoculated mixed cultures tend to resemble 'black boxes': it is hardly possible to interpret their response in terms of environmental or inter-strain influences. In order to simplify the system, the experiments tabulated in Tables 3 and 4 were designed in two consecutive stages with the aim of creating exaggerated situations of nutrient depletion and/or accumulation of inhibitory substances during the first stage. The tables show the response by the strains inoculated during the second stage.

Data presented in Table 3 indicate that competition for carbon rather than nitrogen by *Rhizopus* 581 and *Neurospora* 429 plays a role in suppressing the aflatoxin B_1 accumulation. However, it is clear that biomass production as well as aflatoxin B_1 synthesis were inhibited by heat-stable compounds produced by *Rhizopus* 581 and *Neurospora* 429 as pure cultures in GE. The sterilization of the medium prior to inoculation with *Aspergillus* 013 rules out the activity of aflatoxin B_1-degrading enzymes of *Rhizopus* or *Neurospora* origin. On the other hand, it has been reported (Bean & MacFall, 1982) that certain organic acids including fumaric acid inhibit aflatoxin biosynthesis; these may well have been present in the cultures.

The ability to degrade aflatoxin B_1 by *Rhizopus* 581 and *Neurospora* 429 as shown in Table 4 adds to the complex nature of the self-protection of fermented foods as mentioned earlier by Ko (1974). The formation of various fluorescent substances from pure aflatoxin B_1 by both *Rhizopus* 581 and *Neurospora* 429 and the degradation of aflatoxin B_1 by *R. oryzae* reported earlier (Jarvis, 1971; Cole, Kirksey & Blankenship, 1972) confirm their ability to metabolize aflatoxin B_1. However, the nature of the degradation products and their possible toxicity were not investigated.

Apart from the ability of producer strains to degrade aflatoxin B_1 reported elsewhere (Doyle & Marth, 1978; Huynh & Lloyd, 1984), this investigation shows that in mixed fungal communities competition for carbon sources, inhibition of growth and aflatoxin B_1 biosynthesis, as well as aflatoxin B_1 degradation by other than producer strains may take place simultaneously, with the overall reduction of aflatoxin B_1 accumulation.

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