In vitrointeraction of actinomycetes isolates withAspergillus flavus: impact on aflatoxins B1 and B2 production

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In vitro interaction of actinomycetes isolates with *Aspergillus flavus*: impact on aflatoxins B1 and B2 production

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Significance and Impact of the Study: Interaction between *Aspergillus flavus* and Actinomycetes isolates was conducted *in vitro*. Actinomycetes isolates having a mutual antagonism in contact with *A. flavus* were chosen for further aflatoxins production study. This is a new approach based to develop biocontrol against aflatoxins accumulation in maize while respecting natural microbial equilibrium.

Abstract

This work aimed to study the interaction between Actinomycetal isolates and *Aspergillus flavus* to promote mutual antagonism in contact. Thirty-seven soilborn *Streptomyces* spp. isolates were chosen as potential candidates. After a 10-day *in vitro* co-incubation period, 27 isolates respond to the criteria, that is, mutual antagonism in contact. Further aflatoxins B1 and B2 analysis revealed that those 27 isolates reduced aflatoxin B1 residual concentration from 38·6 to 4·4%, depending on the isolate. We selected 12 isolates and tested their capacity to reduce AFB1 in pure culture to start identifying the mechanisms involved in its reduction. AFB1 was reduced by eight isolates. The remaining AFB1 concentration varied between 82·2 and 15·6%. These findings led us to suggest that these eight isolates could be used as biocontrol agents against AFB1 and B2 with low risk of impacting the natural microbial equilibrium.

Introduction

Aflatoxins B1 (AFB1) and B2 (AFB2) (AFBs) are secondary metabolites produced by filamentous fungi *Aspergillus flavus*. Aflatoxins are carcinogenic compounds (IARC 2012), and their presence in food is a major food-related health issue. Aflatoxins are commonly found in foodstuffs such as groundnuts, wine, maize and feed products such as wheat (Magan *et al.* 2011). Maize has the highest risk of aflatoxin contamination among cereals in the European Union (Piva *et al.* 2006), which has limited its presence to 4 μg kg^{-1} in maize foodstuff (European Union 2006).

Several studies have analysed aflatoxin contamination in maize. Each step of the cereal food chain can be affected by aflatoxin contamination. Contamination can be controlled at seed, field, production, storage or food processing levels (Abbas *et al.* 2009; Elsanhoty *et al.* 2013). At the field level, this control can be made by jointly observing weather settings [water activity (aw), temperature, etc.] and acting upon agricultural practices (irrigation, fertility, reinforced insects prevention, etc.) (Abbas *et al.* 2009).

In addition, biocontrol agents are able to reduce AFB1 accumulation. Two similar biocontrol agents are already commercialized against AFB1 accumulation: afla-guard® (Circle One Global, Inc., Shellman, GA) and afla-safe®(IITA, Ibandan, Nigeria). These nonaflatoxigenic *A. flavus* strains can prevent aflatoxins occurrence between 70·1 and 99·9% by competing and displacing aflatoxin producers (Atehnkeng *et al.* 2008). This displacing overtakes the maize fungal niche and prevents other mycotoxigenic fungi to colonize maize. This shows that fungus contamination is widely tolerated as long as there is no aflatoxin production (Atehnkeng *et al.* 2008). No other micro-organism is currently available to avoid aflatoxin accumulation in maize.

Other micro-organisms are being tested for their action on AFB1 accumulation or degradation/removal. In terms of preventing accumulation, Sultan and Magan (2011) and Zucchi *et al.* (2008) showed how actinomycetes genera—*Streptomyces* can inhibit AFB1 production *in vitro*. Other bacteria are also being tested as agents for AFB1 degradation or removal: *Nocardia corynebacteroides* (Tejada-Castañeda *et al.* 2008), *Enterococcus faecium* (Topcu *et al.* 2010), *Flavobacterium aurantiacum*, *Mycobacterium fluoranthenivorans* and *Corynebacterium rubrum* have been shown to efficiently detoxify AFB1 (Wu *et al.* 2009). Lactic acid bacteria have been shown to reduce AFB1 concentration by 45% thanks to cell wall surface binding (El-Nezami *et al.* 1998). Another described mechanism is the enzymatic degradation of *Rhodococcus erythropolis* (Alberts *et al.* 2006), *Peniophora*, *Pleurotus ostreatus* and *Trametes versicolor* (Alberts *et al.* 2009). However, there is no usable biocontrol which can both prevent accumulation in the field and remove AFB1.

In this context, our work was planned to screen actinomycetes for their ability to do mutual antagonism at contact with *A. flavus*. The corresponding actinomycetes isolates were investigated for AFBs production reduction. The best performing strains were put in an AFB1-supplemented media and further analysed.

Results and discussion

Actinomycetes were chosen as micro-organisms to be tested due to their tolerance to water stresses, their broad spectrum of metabolite production and survival in most soils and crops (de Araújo *et al.* 2000; Doroshenko *et al.* 2005).

Actinomycetes isolates selection

The 37 strains grew well on ISP-2 medium. They formed nonfragmented and colourless or yellowish brown substrate mycelium. The aerial mycelium was grey, yellowish grey or greyish yellow and produced numerous nonmobile and straight or spiral spore chains carried by sporophores. Diffusible pigments were not observed. This description corresponded to the genus *Streptomyces* (Holt *et al.* 1994).

Screening results

Thirty-seven actinomycete candidates were chosen for screening. After 10 days on ISP-2 medium, three patterns were observed (Table 1). Four of the 37 tested isolates showed an I_D (0/5) that represents an 'actinomycete dominance at distance' pattern and can decrease AFB residual concentration in the medium (rcm). Each of these four

Table 1 Actinomycetes presenting the same I_D and impact on AFBs concentration

I_D	Number of strains	AFBs concentration	Number of strains	AFBs rcm in % range
(0/5)	4	Reduction	4	4·4–9·7
		No reduction	0	
(4/0)	6	Reduction	4	8·1–46·1
		No reduction	2	
(2/2)	27	Reduction	27	4·4–38·6
		No reduction	0	

I_D = Applied to *A. flavus*/actinomycete strain (as defined in *Materials and methods*) reduction = AFBs concentration reduction compared with the control no reduction = AFBs concentration equivalent to control ($P < 0.05$).

isolates (S25, S26, S31 and S36) individually inhibits *A. flavus* growth at distance. S25 has the most impact on AFB1 and AFB2 rcm (4·4 and 5·3%).

Among the 37 isolates, six showed an I_D (4/0) that represents a 'dominance of *A. flavus* on actinomycete isolate' pattern (Table 1). For these isolates, macroscopic observations revealed that *A. flavus* completely covered the actinomycete inoculation streak. Regarding AFBs, we observed that S15 had no impact on both AFBs rcm. S7, S12, S24 and S37 presented lower AFBs rcm. The maximum decrease in AFBs content was observed in the presence of S12 or S37 isolates with a rcm of 8·1 and 9·1%, respectively, for AFB1 and of 9·6 and 10·2% for AFB2.

The 27 remaining isolates showed an I_D (2/2) that represents 'mutual antagonism on contact' and promotes both micro-organisms growth. They can reduce AFBs rcm from 38·6 to 4·4% rcm.

We were able to demonstrate different I_D patterns from Sultan and Magan (2011) (Table 1). Indeed, they demonstrated that among the six Egyptian *Streptomyces* tested, five had mutual intermingling with *A. flavus* (I_D 1/1) and one had dominance at a distance (I_D 5/0). However, this can be explained by the previous selection done in our study. Little data are available on *Streptomyces–Aspergillus* micro-organisms interaction, because many studies have focused on *Streptomyces* free cell extracts. Mohamed *et al.* (2013) tested 16 Egyptian rhizosphere *Streptomyces* for their potential antagonism. They revealed that 69% of free cell extracts were not able to reduce fungal growth.

Those studies are showing that a big part of the tested *Streptomyces* are not able to reduce fungal growth. Unlike our study, other researchers have chosen fungal growth inhibition as the first selection criteria for their potential biocontrol agents against mycotoxin production (Sultan and Magan 2011; Haggag and Abdall 2012). Our work focuses on promoting both micro-organisms growth: I_D (2/2).

I_D (2/2)—mutual antagonism on contact

Twenty-seven isolates showed an I_D (2/2) that represents 'mutual antagonism on contact'. The results are presented in Table 2. They are classified from the lowest to the highest impact of specific actinomycete isolates on AFB1 rcm.

We observed that when *A. flavus* is in contact with those actinomycetes streaks, fungal growth is slightly reduced (about 30%). All the 27 isolates reduced AFB rcm compared with the control. S10–S11 (Table 2) showed a slight decrease in AFB1 concentration, particularly with isolate S10 preserving 38·6% rcm. We focused on S3–S35 in Table 2, which have the highest AFB1 rcm reduction.

Co-culture of each isolates from S3 to S17 (as shown in Table 2) had a AFB1 rcm above 10%. In addition, S38–S35 led to an efficient decrease of AFB1 rcm (<10%). In this last group, a co-culture of S38 showed the lowest level of AFB1 reduction. S35 was the most efficient in reducing the amount of AFB1 (rcm of 4·4%).

Focusing on AFB2, we observed that rcm varied between 27·1 and 10·5% for S3–S35 (Table 2). Besides, isolates S23 and S22 were less efficient in AFB2 than AFB1 reduction. If only AFB2 reduction is considered, S35 is again the most efficient candidate (rcm of 5·5%).

Overall, isolates with an AFB1 and AFB2 rcm of <17% (after S11 in Table 2 except S23 and S22) could be interesting candidates for further studies. Considering the decrease of both aflatoxin levels, we concluded that S1, S6, S27 and S35 were effective in co-culture with *A. flavus*. This aflatoxin reduction may be linked to the actinomycete metabolites.

Ono *et al.* (1997) identified a molecule produced by *Streptomyces* sp. MRI142 called aflastatin A which is able to completely inhibit AFB1 production at 0·5 μg ml^{-1} without affecting fungal growth. Thus, our results could be linked with a possible production of aflastatin A by our candidate isolates. Another *Streptomyces* molecule called Dioctatin A was identified by Yoshinari *et al.* (2007). This molecule inhibits *Aspergillus parasiticus* conidiogenesis and AFB1 production. These results are different from our results showing no conidiogenesis macroscopic impact for the 27 isolates tested. Thus, it is unlikely that our results are linked with actinomycetes Dioctatin A.

Focusing on biological control approaches already applied in the field, Nigerian nontoxigenic strains of *A. flavus* were able to reduce to 0·02% AFB1 content *in vitro* on maize kernel (Atehnkeng *et al.* 2008). These results were the first step towards Afla-safe® commercialization. The main criterion in the atoxigenic strains selection was the capacity to outmatch toxigenic strains. Comparatively, we have promoted the isolates that can grow in contact with *A. flavus*. Thus, the actinomycete growth in *A. flavus* presence was monitored.

Actinomycetes growth in *A. flavus* presence

For the 27 isolates in mutual antagonism on contact with *A. flavus*, actinomycete growth was measured to select isolates able to develop when on contact with *A. flavus*. We observed that some of the isolates grew more efficiently than others in *A. flavus* presence. For instance, S10, S30 and S34 had the smallest spread with seven mm. Twenty-one isolates were able to spread between 0·9 and

Table 2 Effect of different actinomycetes isolates on fungal growth and aflatoxin B1 and B2 concentration. Only the 27 which has shown mutual antagonism on contact with *Aspergillus flavus* (I_D(2/2) are represented

Strain	Fungal growth (%)	AFB1 (rcm in%)	AFB2 (rcm in%)	Strain	Fungal growth (%)	AFB1 (rcm in%)	AFB2 (rcm in%)
Control	100·4 ± 1·7[a]	100·5 ± 5·5[a]	100·9 ± 9·4[a]	S8	69·6 ± 4·0[b]	15·1 ± 1·3[c]	16·0 ± 3·7[c]
S10	72·1 ± 4·2[b]	38·6 ± 15·4[b]	33·2 ± 18·1[b,c,d]	S16	67·8 ± 1·4[b]	14·8 ± 2·7[b,c,d]	16·6 ± 2·6[b,c,d]
S18	79·9 ± 22·1[b]	25·8 ± 6·6[b]	20·6 ± 1·4[b]	S4	70·0 ± 4·1[b]	14·6 ± 2·5[b,c,d]	15·8 ± 4·3[c]
S5	87·4 ± 14·8[b]	23·7 ± 8·5[b]	29·7 ± 4·7[b]	S34	71·1 ± 3·1[b]	12·8 ± 2·1[c,d]	15·2 ± 4·7[b,c,d,e]
S20	64·2 ± 1·7[b]	22·5 ± 5·7[b]	22·4 ± 6·4[b,c]	S33	77·0 ± 11·1[b]	11·9 ± 9·8[b,c,d,e]	13·9 ± 10·6[b,c,d,e]
S29	70·7 ± 2·7[b]	20·4 ± 4·0[b,c]	21·1 ± 16·1[b,c,d]	S2	68·7 ± 2·0[b]	11·2 ± 1·9[d]	10·5 ± 3·6[d,e]
S30	65·1 ± 1·3[b]	20·0 ± 8·2[b,c,d]	16·1 ± 6·8[b,c,d,e]	S17	67·7 ± 1·4[b]	10·6 ± 1·4[d]	10·8 ± 2·0[b,c,d,e]
S19	67·4 ± 1·3[b]	20·8 ± 6·8[b,c]	25·8 ± 6·4[b,c,d]	S38	74·7 ± 13·1[b]	8·8 ± 3·8[c,d,e]	13·0 ± 3·5[b,c,d,e]
S14	66·4 ± 0·8[b]	18·2 ± 5·5[b,c,d]	16·7 ± 3·6[b,c,d]	S13	87·1 ± 12·1[b]	7·9 ± 2·2[d,e]	8·3 ± 3·0[b,c,d,e]
S11	68·3 ± 2·8[b]	18·0 ± 1·2[b]	18·6 ± 1·9[b,c]	S27	71·5 ± 11·6[b]	7·0 ± 1·4[e]	8·1 ± 2·0[d,e]
S3	71·4 ± 3·6[b]	16·9 ± 3·0[b,c,d]	17·0 ± 0·9[c]	S28	66·8 ± 0·6[b]	6·9 ± 1·9[d,e]	10·7 ± 2·1[b,c,d,e]
S22	65·9 ± 1·6[b]	16·1 ± 2·0[b,c]	27·1 ± 3·5[b]	S1	72·6 ± 8·1[b]	6·2 ± 0·8[e]	8·7 ± 1·6[d,e]
S21	69·8 ± 2·5[b]	15·8 ± 3·5[b,c,d]	14·2 ± 2·6[b,c,d]	S6	67·9 ± 5·1[b]	5·9 ± 2·1[e]	7·3 ± 2·1[e]
S23	70·9 ± 2·1[b]	15·8 ± 2·3[b,c]	20·4 ± 2·5[b,c]	S35	69·1 ± 3·3[b]	4·4 ± 1·0[e]	5·5 ± 1·3[e]

Data with the same letter are not significantly different (P < 0·05).

1·7 cm. S18 and S6 show the most significant spreading with 1·7 and 1·8 cm, respectively.

Effects of selected actinomycetes isolates on pure AFB1

Thirty-seven isolates were screened for their ability to reduce AFB rcm without having an impact on fungal growth. We revealed that 27 isolates were corresponding to those criterions. Among these, 12 of the most efficient reducers were chosen for further characterization. We decided to test whether they reduce AFB1 concentration in pure culture to start identifying the mechanisms involved in its reduction.

The 12 selected actinomycetes isolates were inoculated in the presence of AFB1 in solid media at a concentration of 5 mg kg^{-1}. Results are shown in Table 3.

Among the 12 chosen actinomycetes, seven showed no macroscopic difference when AFB1 was present in the medium (represented by the minus symbol in Table 3). The remaining five isolates showed phenotypic differences. We observed a lack of white pigmentation for S35 and S38 (linked to sporulation) in the presence of AFB1. S6 and S27 showed a reduction in streak width. S27 had a 1 mm streak width instead of 4 mm in the control, and S34 did not grow in the presence of AFB1.

The AFB1 level in the media was analysed for each of these 12 isolates. Results are shown in Table 3. S13, S17 and S34 had no impact on the AFB1 rcm. S8, S21, S27 and S33 slightly reduced the initial AFB1 concentration (rcm between 82·2 and 69·8%), even if S35 and S38 showed a more significant reduction in the AFB1 concentration (rcm of 29·4 and 38·0%, respectively). Finally, S3, S4 and S6 were extremely efficient in the reduction of AFB1 concentration (rcm: 22·2, 27·3 and 15·6%, respectively). Joining co-culture and pure AFB1 test results, we

can see that S3 reduced AFB1 rcm (16·9%), and this is due to the isolate degradation or adsorption properties. In contrast, S17 reduced AFB1 rcm (10·6%) in co-culture but had no impact in contact with pure AFB1. This could be linked to other mechanisms like aflatoxin biosynthesis inhibition.

In summary, S3, S4, S6 and S35 showed a rcm above 30% and are potential candidates for the reduction of AFB1 concentration. Among these, only S6 and S35 showed a differential phenotype in the presence of AFB1.

For the 12 selected isolates, HPLC chromatograms were investigated. Only three (S3, S4 and S6) revealed a peak emergence in their chromatogram profile. We presumed that this peak is due to aflatoxin degradation or actinomycete metabolites production in response to AFB1 presence. This could be linked to the presence of partially hydrophobic lower molecular weight molecules (shorter retention time) as a result of AFB1 degradation. Taylor *et al.* (2010) demonstrated that the $F_{420}H_2$ reductase is able to reduce AFB1 α,β-unsaturated ester moiety, which resulted in several low molecules appearance. This reductase commonly found in *Actinomycetales* genus has not yet been characterized in *Streptomyces* genus (Purwantini *et al.* 1997).

Many studies have attempted to control the toxicity of AFB1 disruption by-products. Megalla and Hafez (1982) demonstrated that AFB1 can be converted to less toxic derivates such as aflatoxin B_{2a}. Recently, Samuel *et al.* (2014) showed that *Pseudomonas putida* can biotransform AFB1 to less toxic compounds, aflatoxins D. Other studies reported that AFB1 can be degraded by *Pseudomonas* spp. and other soilborn bacteria. The degradation results revealed a toxicity reduction compared with control sample (Elaasser and El Kassas 2011; Krifaton *et al.* 2011). These results imply the prospective that our isolates could detoxify the medium. However, they must be investigated for biosafety tests.

Our study has demonstrated that actinomycetes can reduce *in vitro* A. *flavus* aflatoxins accumulation without impacting fungal growth. We have also shown that actinomycetes can reduce AFB1 concentration in solid media. Our study is the first step in developing actinomycetes as biocontrol agents against AFB1 on maize grain. Further mechanistic approaches can be done focusing on the impacts of our potential biocontrol candidates on aflatoxins G1 and G2 producer A. *parasiticus*.

Materials and methods

Fungal strain and actinomycete isolates

The fungal strain used was A. *flavus* NRRL 62477. Actinomycetes strains were collected from soils of different

Table 3 Impact of actinomycetes on aflatoxin B1 concentration in the media

Strain	AFB1 impact on actinomycetal growth	AFB1 (rcm in%)
Control	/	100·0 ± 2·2[a]
S13	–	104·9 ± 17·0[a]
S17	–	96·6 ± 17·1[a,b]
S34	+	92·7 ± 8·5[a,b]
S21	–	82·2 ± 6·0[b]
S8	–	78·1 ± 15·2[b]
S27	+	76·6 ± 13·7[b]
S33	–	69·8 ± 11·5[b]
S38	+	38·0 ± 11·1[c]
S35	+	29·4 ± 15·2[c]
S4	–	27·3 ± 2·2[d]
S3	–	22·2 ± 5·4[d]
S6	+	15·6 ± 11·7[d]

Data with the same letter are not significantly different ($P < 0.05$).

locations in Algeria (Adrar, Biskra, Ghardaïa, Hassi R'Mel and Laghouat) by a dilution agar plating method using chitin–vitamin agar medium (Hayakawa and Nonomura 1987) supplemented with cycloheximide (80 mg l^{-1}) and nalidixic acid (15 mg l^{-1}) to suppress the growth of fungi and Gram-negative bacteria, respectively. A first antagonism test was realized against *A. flavus,* and 37 strains showing the less antagonistic characteristics were selected for screening and numbered from 1 to 38. The cultural characteristics of actinomycete strains were observed by naked-eye examination of 14-day-old cultures grown on yeast extract/malt extract agar (ISP-2) medium (Shirling and Gottlieb 1966). Spores and mycelium were examined by light microscopy (Motic; B1 Series). They were conserved at −20°C in cryotubes in a 20% glycerol solution.

Culture media

Precultures of *A. flavus* were inoculated on YEPD medium containing 5 g l^{-1} yeast extract, 10 g l^{-1} casein peptone, 10 g l^{-1} α-D-glucose and 15 g l^{-1} agar. Actinomycete isolates precultures were inoculated on ISP-2 medium (Shirling and Gottlieb 1966) at pH 7. The

micro-organisms were preincubated separately on ISP-2 (actinomycetes) and YEPD (*A. flavus,* when needed), at 28°C for 7 days.

Co-culture screening method

The co-culture screening method is based on the method proposed by Sultan and Magan (2011). *A. flavus* spores were dislodged from the preculture with a sterile loop and placed in 10 ml sterile water +0·05% Tween-20. In a Petri dish filled with ISP-2 medium, actinomycetes and *A. flavus* were inoculated on the same day as described in Fig. 1. The spore suspension from *A. flavus* was spotted, and the actinomycetes were inoculated with a streak. The incubation lasted 10 days at 28°C, and growth measurements were carried out at the end of the incubation period. The experiment was realized twice in triplicate. The interaction between the two micro-organisms was observed macroscopically and scored based on the Index of Dominance (I_D) (Magan and Lacey 1984). The I_D is determined by addition of individual scores based on: mutual intermingling (1/1), mutual antagonism on contact (2/2), mutual antagonism at a distance (3/3), dominance of one species on contact (4/0) or dominance
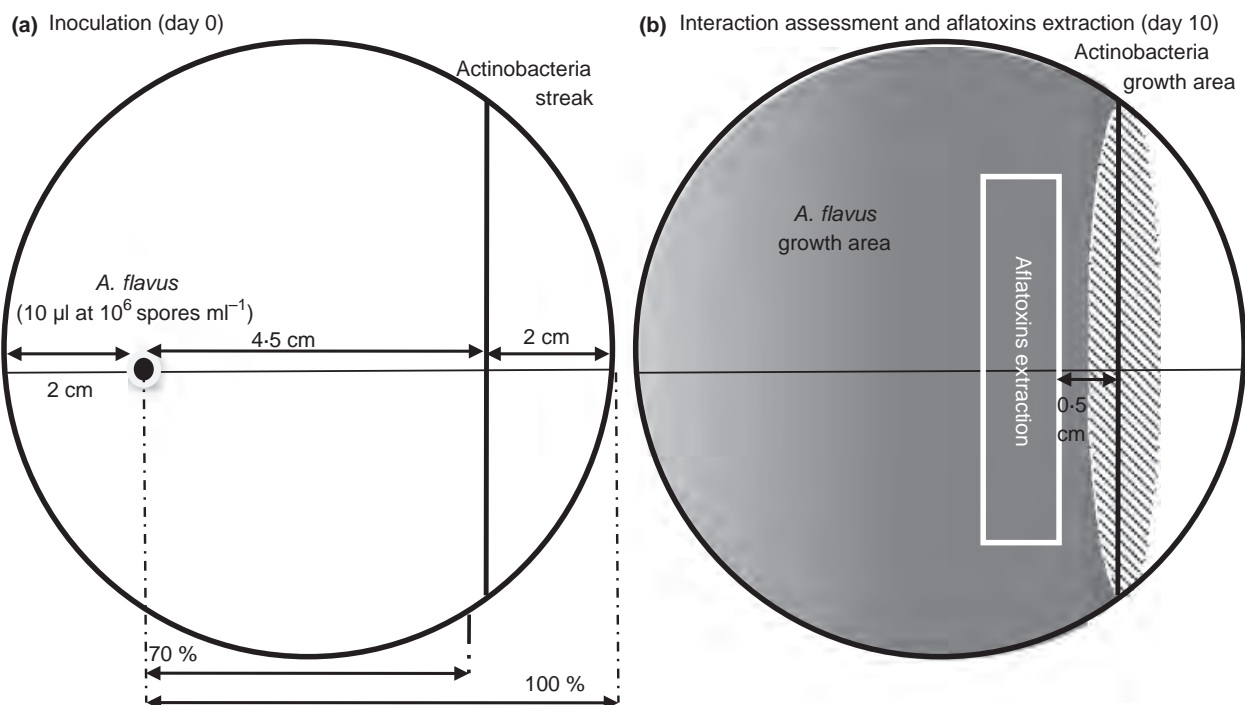

Figure 1 Methodology used for interaction assessment and aflatoxins extraction. (a) Inoculation (day 0) in a Petri dish filled with ISP2 medium, actinomycetes and *A. flavus* are inoculated on the same time. Inoculation is done with the following instructions: 10 μl of spores suspension from *A. flavus* is spotted at 2 cm from the Petri dish periphery. Actinomycete streak is inoculated perpendicularly to *A. flavus*–actinomycete axe at 4·5 cm of the *A. flavus* spot. (b) Interaction assessment and aflatoxins extractions, in case of ID (2/2). The growth measurements are carried out and are represented in grey for *A. flavus* and in stripes for the isolate. The aflatoxin extraction area is delimited by a white box.

at a distance (5/0) occurred for each *A. flavus*/actinomycetes interaction.

Solid media AFB1 reduction test

A 1 mg ml^{-1} AFB1 solution was prepared in methanol solution. This solution was added to ISP2 medium after autoclaving to obtain a final concentration of 5 mg kg^{-1}. Actinomycetes were inoculated with a loop to cover completely the Petri dish surface. After a 4 days long incubation period at 28°C, AFB1 was extracted as described below. The actinomycete growth was observed macroscopically in the control media (without AFB1) and in the AFB1-supplemented media. The experiment was realized twice in triplicate.

Aflatoxins extraction

Three agar plugs (Ø 9 mm) were taken both 5 mm away from actinomycete streak for the co-culture screening method and randomly on actinomycete growth area for the solid media AFB1 reduction test.

The total weight was measured. One millilitre of methanol was added to the plugs and shaken for 5 s three times. After 30-min incubation at room temperature, solutions were centrifuged 15 min at 12 470 **g**. The supernatant was taken and filtered through 0·45-μm PVDF Whatman filter into vials and stored at −20°C until analysis. The recovery ratio was 50%.

AFB1 and AFB2 detection and quantification by HPLC

The HPLC system used for aflatoxins analysis was an Ultimate 3000 system (Dionex–Thermo Fisher Scientific, Courtaboeuf, France) with all the RS series modules. A C18 column and its associated precolumn (Phenomenex, Luna 3 μm, 200 × 4·6 mm) was used. The mobile phase and AFB1 derivatization were realized according to the Coring Cell$^{®}$ instruction (Coring System Diagnostix Gmbh, de). Analyses were realized at a flow rate of 0·8 ml min^{-1} during a 35-min run. The quantification was realized by the Chromeleon software, thanks to standards of AFB1 and AFB2 (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The limit of quantification is 0·5 ppb for each.

Statistical analysis

The statistical analyses were performed with R (2.15.2; Lucent Technologies, Auckland, Australia) for nonparametric events, the package 'nparcomp' was used and the contrast method was Tukey with a confidence level of 95% and a logit transformation.

Conflict of interest

No conflict of Interest declared.

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