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Biocontrol of *Aspergillus flavus* Producing Aflatoxin B1 by *Streptomyces exfoliatus*

Abd El-Raheem R. El-Shanshoury[(1)#], Metwally A. Metwally[(1)], Sabha M. El-Sabbagh[(2)], Hamdy A. Emara[(3)], Heba allah E. Saba[(1)]

[(1)]Microbiology Section, Botany and Microbiology Department, Faculty of Science, Tanta University, Tanta 31527, Egypt; [(2)]Botany Department, Faculty of Science, Menoufyia University, Shebin El-Koom, Egypt; [(3)]Microbiology Department, Soil, Water, and Environment Institute, Agriculture Research Center, Giza, Egypt.

IN tropical and subtropical regions, contamination of crops by aflatoxin B1 (AFB1) is a growing challenge. To restrict fungal growth and allow aflatoxin detoxification, there is a need to control mycotoxin contamination. *Streptomyces exfoliatus* has shown promise against several phytopathogenic fungi. This study aimed to explore whether *S. exfoliatus* could be employed as a biocontrol agent against *Aspergillus flavus* and AFB1 contamination. In this study, the biological control of AFB1 production by *A. flavus* was examined using some actinobacteria isolates. The cytotoxic activity of AFB1 and its degradation products were also evaluated. Results revealed that the most effective actinobacterium against *A. flavus* was isolate number 1, which was identified as *S. exfoliatus*. The growth, sporulation, and AFB1 production of *A. flavus* decreased when it was treated with a cell-free culture filtrate produced by the identified isolate. Furthermore, 20% of the cell-free culture filtrate of *S. exfoliatus* completely inhibited AFB1 production. The AFB1 content was also reduced in wheat samples treated with day intervals. After 21 days, no AFB1 was detected compared with those of the controls. After 3 days, 95.47% of AFB1 was degraded by the cell-free culture filtrate. The degraded AFB1 products were less toxic than the parent aflatoxin. The optimum temperature of AFB1 degradation was 30°C, but AFB1 degradation decreased as the temperature further increased. The cell-free culture filtrate remained stable for 12 months at the time of freezing. In conclusion, *S. exfoliatus* cell-free culture filtrates can inhibit the growth and sporulation of *A. flavus* as well as reduce AFB1 generation and degrade it into less harmful compounds. This appears a promising option for minimizing contamination by *A. flavus*, preventing aflatoxin accumulation, and enabling aflatoxin breakdown in wheat grains; this approach may be used to reduce *A. flavus* and AFB1 contamination in future biological control programs.

Keywords: *Aspergillus flavus*, Aflatoxin B1, Cell-free culture filtrate, Degradation, *Streptomyces exfoliates*.

Introduction

The food intended for human and animal consumption must be free of contaminants to minimize or prevent not only disease onset, poisoning, and death (FAO, 2002) but also economic losses (Cardwell et al., 2001). Several strategies, such as preventing contamination during food production, processing, and storage, should be strictly implemented to achieve these objectives, especially in the case of mycotoxins (IARC, 2002).

Fungal species common in grains include *Fusarium verticillioides*, *F. proliferatum*, *Aspergillus flavus*, *A. ochraceus*, *A. parasiticus*, *Penicillium verrucosum*, *P. commune*, and *Blumeria graminis* (CAST, 2003; Rustemeyer et al., 2010; El-Shanshoury et al., 2014; Ashiq, 2015; Benkerroum, 2020; Rózewicz et al., 2021). These

[#]Corresponding author emails: abdelreheam.elshanshoury@sience.tanta.edu.eg, abrabsh@gmail.com
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fungal species produce mycotoxins that pose a serious toxicological risk to animals and humans. The main mycotoxins are aflatoxins, ochratoxin, patulin, trichothecenes, and fumonisins. Aflatoxins contaminate agricultural products such as corn, wheat, rice, peanuts, and many other crops (Yin et al., 2008; Ashiq, 2015; Mahato, 2019; Jallow et al., 2021). In addition, aflatoxin B1 (AFB1) is positively correlated with liver cancer, acute and chronic poisoning, and death in humans and animals (Azziz-Baumgartner et al., 2005; Seo et al., 2011; Benkerroum, 2020).

Aflatoxins can be controlled using various methods that can be classified into two categories: (1) The prevention of mold contamination and growth and (2) The detoxification of contaminated products (Pleadin et al., 2014; Lavkor & Var, 2017; Benkerroum, 2020). Likewise, AFS contamination can be prevented through different strategies that are generally divided into two categories: pre- and post-harvest controls (Kabak et al., 2006; Lavkor & Var, 2017). Control strategies before harvesting are carried out mainly by practicing appropriate field management (such as crop rotation, irrigation, and soil cultivation), producing AF-resistant crop varieties, applying fungal and bacterial inhibitors, and using biological, natural, and chemical agents. Control strategies after harvesting are based on the improvement of drying conditions and storage through biological, chemical, and natural irradiation (Varga et al., 2010; Agriopoulou, 2020).

A wide range of organisms, including bacteria, yeasts, actinobacteria, algae, and nontoxigenic strains of *A. flavus* and *A. parasiticus* (Dorner, 2004; Yin et al., 2008; Almatakeez, 2020), have been tested for the biological control of aflatoxin contamination in crop in pre- and post-harvest (Halasz et al., 2009; Aliabadi et al., 2013; Peles et al., 2021).

Actinobacteria are potent producers of different secondary metabolites (Landwehr et al., 2016). They have been evaluated as biological control agents against various plant pathogens (Chung et al., 2005; Dewi et al., 2015; Djebail, 2021). Among them, *Streptomyces* species are considered economically important because they comprise only 50% of the total population of soil actinomycetes (Xu et al., 1996) and 75% of total bioactive molecules (Berdy, 2005). For example, *S. exfoliatus* produces tetradecahydrophenazine diphenethyl-2,8-dicarboxylate that exhibits an inhibitory activity against *A. flavus*, *A. niger*, *Fusarium oxysporum*, and *Candida albicans* (El-Sabbagh et al., 2013).

Zucchi et al. (2008) and Sultan & Magan (2011) showed how actinomycetes of *Streptomyces* can inhibit the *in vitro* production of AFB1. In particular, *Streptomyces yanglinensis* 3-10 inhibits the mycelial growth, sporulation, conidial germination, and aflatoxin biosynthesis gene expression of *A. flavus* and *A. parasiticus in vitro*. In peanut kernels, their metabolites reduce disease severity and inhibit aflatoxin production under storage conditions (Lyu, 2020). The growth inhibition of *A. flavus* and AFB1 have been also decontaminated by *Streptomyces* isolates and their metabolites (Campos-Avelar et al., 2021). As key components of biological control methods, various metabolites from *Streptomyces* spp. (Dioctatin A, aflastatin A, and blasticidin), *Achromobacter xylosoxidans* [cyclo (L-leucyl-L-propyl)], and *Bacillus subtilis* (D bacillomycin, iturin A, etc.) have been introduced as potent inhibitors of AFB1 biosynthesis under laboratory conditions (Holmes et al., 2008).

AFB1 reduction by bacteria has been reported. Most studies have focused on lactic acid bacteria, such as strains belonging to Lactobacillus (Rawal et al., 2014; Kurhan & Çakir, 2016), Bifidobacterium (Peltonen et al., 2001), and Propionibacterium (El-Nezami et al., 2000). However, the AFB1 reduction by these bacteria primarily involves binding or metabolism rather than cell degradation. AFB1 is effectively degraded by *Nocardia corynebacteroides* (Wu et al., 2009) and *Enterococcus faecium* (Topcu et al., 2010). In addition, Teniola et al. (2005) and Alberts et al. (2006) reported the AFB1 degradation by *Rhodococcus erythropolis* liquid cultures and intracellular extracts prepared from the liquid culture. They observed that AFB1 is degraded through a cascade of enzymatic reactions with the loss of fluorescence over time. Garai et al. (2021) showed that *R. erythropolis* NI1 strain can degrade mycotoxins and their mixtures in different proportions. They also found that the ratio of mycotoxins significantly inhibited in combination is higher than that in single ones.

This study was conducted to prevent or inhibit *A. flavus* growth and AFB1 production by using a biological method. *Streptomyces* strain was isolated from soil, and its ability to produce

extracellular anti-AFB1 metabolite (s) against *A. flavus* was examined. The effects of the culture filtrates of this antagonist on the growth of *A. flavus*, and its production and degradation of AFB1 *in vitro* and *in vivo* were also investigated.

Materials and Methods

Source of microorganisms

Test actinomycetes were isolated from soil samples collected from different localities in Tanta (El-Gharbia Governorate) and El-Mansura City (Dakahlia Governorate), Egypt. The soil samples were taken after approximately 5 cm of the surface was removed and then kept in clean plastic bags. On the surface of solidified starch nitrate agar plates, 0.2 ml of the samples of soil dilution was spread with a sterile glass rod. The plates were then incubated at 30°C for 7 days. *A. flavus* was isolated from maize, rice, wheat, and peanut samples collected from different locations in Egypt.

Extraction and purification of AFB1

Aflatoxins were extracted from *A. flavus* and food samples in accordance with Association of Analytical Chemists (A.O.A.C., 1984). The final extracts were purified in accordance with previously described methods (Takeda et al., 1979; Endre et al., 2019) with some modifications.

Detection and determination of AFB1 by thin-layer chromatography

Thin-layer chromatography (TLC) was conducted to identify and estimate the quality and quantity of AFB1 in the sample extract (FAO & UNEP, 1989; FAO, 1990). A precoated 60 F254 TLC type without a fluorescent indicator (20cm × 20cm, 0.2mm layer thickness; E, Merck, Germany) was used. A mixture of chloroform:acetone (9:1, v/v) was added to a glass jar with thin-layer silica gel plates to separate AFB1 (supplied by Sigma Chemical Company, USA). The plates were removed from the jar, and AFB1 was examined under exposure to short and long-wavelength ultraviolet (UV) light (254 and 366nm). Rfs samples were compared with those of the standard, and AFB1 showed blue fluorescence. For confirmation, a plate was sprayed with a fine mist of 50% (v/v) H_2SO_4 solution in water and viewed under long-wavelength UV, revealing that AFB1 produced yellow fluorescence. The quantities of AFB1 were determined by quantitatively reading the silica gel plates with a fluorodensitometer (TLD-100 Vitatron) in accordance with the methods described by Shannon et al. (1983) and FAO (1990).

Biological control

The antagonistic activity of the actinomycete isolated against *A. flavus* was detected through diffusion in agar wells (Holmalahti et al., 1994). The isolated actinomycetes were grown in a starch nitrate medium in 250mL Erlenmeyer flasks on a rotary shaker (200rpm) at 30°C for 7 days. The contents of each flask were centrifuged at 6000rpm for 20min, and the supernatant was filter-sterilized with a 0.45µm bacterial filter. Aliquots (50µL) of each sterilized cell-free culture filtrate were applied to wells (5mm diameter), which were made with a sterile cork borer on plates previously seeded with 200µL of 10^6 spores/mL of *A. flavus*. After incubation at 30°C for 3 days, positive findings were noted when the zone of inhibition appeared.

Effect of different concentrations of the cell-free culture filtrate of the selected actinobacterium against A. flavus

Mold was grown in 50mL of Czapek–Dox broth containing yeast extract, dispensed in 250mL Erlenmeyer flasks, and supplemented with the sterilized cell-free culture filtrate to concentrations of 5%, 10%, 15%, 20%, 40%, 60%, 80%, and 100% (v/v) to examine the ability of the cell-free culture filtrate to inhibit the production of AFB1 by *A. flavus*. A control experiment was also performed by adding an equal volume of sterile distilled water instead of the cell-free culture filtrate. Three flasks were prepared for each concentration of the cell-free culture filtrate. Then, each flask was inoculated with 0.1mL of a spore suspension containing 10^6 spores/mL. After incubation at 30°C for 10 days, the dry weight and AFB1 were determined.

The percentage inhibition of aflatoxin production was calculated with the following equation:

$$\text{Inhibition of AFB1 production (\%)} = \frac{AC - AS}{AC} \times 100 \quad (1)$$

where, *AC* is the amount of AFB1 in the control sample, and *AS* is the amount of AFB1 in the treated sample.

In vivo studies on the effect of the selected actinobacterium isolate on A. flavus sporulation and AFB1 production in wheat grains

In this procedure, 15g of aflatoxin-free wheat

grains was placed in Petri dishes and sterilized for two consecutive days in an autoclave at 121°C for 15min. In the treatment (no. 1) plates inoculated with 1mL of 2 × 10^6 CFU/mL of the selected actinomycete isolate and 1 ml of *A. flavus* spores (1 × 10^6 spores/mL) at the same time and in the treatments (nos. 2, 3, and 4) the selected *actinobacterium* isolate no. 1 was inoculated after 7, 14, and 21 days of inoculation of *A. flavus* as mentioned formerly. The positive control set consisting of plates containing wheat was inoculated with the fungus as formerly described, and the plates containing wheat inoculated only with actinomycetes were set as the negative control. The plates were placed in a jar filled with a solution of glycerol to give 0.88 a_w of the whole system and incubated at 25°C for 10 days. AFB1 was determined, and the number of spores was estimated using a hemocytometer; then, the number of spores per gram was calculated (Tzortzakis & Economakis, 2007).

The percent inhibition of spore production was computed with the following equation:

$$\text{Inhibition of spore production (\%)} = \frac{SC - SS}{SC} \times 100 \quad (2)$$

where, *SC* is the number of spores in the control sample, and *SS* is the number of spores in the treated sample.

The percent inhibition of AFB1 production was calculated using equation (1).

AFB1 degradation by the cell-free culture filtrate of actinobacterium isolate no. 1

In this experiment, the standard AFB1 solution (Sigma Chemical Company, USA) was diluted with methanol and added to the cell-free culture filtrate to obtain a final concentration of 5ppm/mL. The mixture was incubated in the dark at 30°C without shaking for 4 consecutive days. After incubation, AFB1 was extracted and determined. AFB1 without cell-free culture filtrate was used as the control.

Effect of temperature on AFB1 degradation by cell-free culture filtrate

In this experiment, the standard AFB1 solution in methanol was added to the cell-free culture filtrate to produce a final concentration of 5 ppm. The mixture was incubated in the dark at 20°C, 25°C, 30°C, 35°C, and 40°C without shaking for 3 days. After incubation, AFB1 was extracted and determined. AFB1 without the cell-free culture filtrate was used as the control.

Effect of shelf life and high temperature on antifungal activity:

The cell-free culture filtrate was kept at high temperatures (40°C, 60°C, 80°C, and 100°C) in glass tubes for 1h to determine the effect of temperature on the stability of the produced antifungal substance. In another treatment, the cell-free culture filtrate was autoclaved at 121°C in glass tubes for 15min. Afterward, the antifungal activity of the cell-free culture filtrate against *A. flavus* was determined.

The effect of shelf life (storage time) on the antifungal activity of the cell-free culture filtrate was determined by storing it in tubes at room temperature, 4°C, and freezing temperature for different periods (2, 4, 6, 8, 10, and 12 months). After the specified period, 50 µl from each tube was added to the wells of Czapek–Dox plates already swabbed with *A. flavus*.

Cytotoxicity test of AFB1 degradation products

This test was conducted in accordance with the modified method of Krishnaraju et al. (2005). The collected nauplii of *Artemia salina* (brine shrimp) were treated with different concentrations of the degraded AFB1. Ten nauplii were drawn through a glass capillary and placed in a 5mL vial containing 4mL of brine solution and 0.5mL of methanolic solution of the degraded AFB1 products. The final volume of 5mL was prepared by adding brine solution. The test at each concentration was performed in triplicate. The experiment was maintained at room temperature for 24h under light conditions, and the surviving brine shrimp larvae were counted. Percentage mortality was determined as the number of dead nauplii divided by the initial number of nauplii (10) multiplied by 100 (Ogunnusi & Dosumu, 2008). Saline and methanol were used as negative controls, whereas 5µg/mL AFB1 was set as the positive control (Sahpaz et al., 1996; Chen et al., 1998).

Characterizations of the selected actinobacterium isolate (no. 1)

Several physical, morphological, and biochemical properties were examined to identify the most fungal and AFB1 antagonistic isolate no 1, in accordance with the identification criteria described in previous studies (Küster, 1972;

Nonomura, 1974; Szabo & Csortos, 1975; Szabo et al., 1975) and Bergey's Manual of Systematic Bacteriology (Williams et al., 1989; Holt et al., 1994).

Results

Eighty isolates of actinobacteria were tested to control the growth of *A. flavus* via the disc diffusion method. Of these isolates, only four had a high antagonistic activity. The antifungal activity of the cell-free extracts of the four tested isolates was determined through agar well diffusion. Table 1 shows that the most potent isolate against *A. flavus* was isolate no. 1. Its effectiveness increased over 8 days of incubation. As a result of the experiment, isolate no. 1 was selected as the experimental organism for further study through the subsequent experiments.

Effect of different concentrations of the cell-free filtrate of the selected actinobacterium on A. flavus

As shown in Table 2, the growth of *A. flavus* significantly decreased when the filtrate concentration increased. AFB1 production also decreased as the antagonistic filtrate concentrations increased until 10%. When the concentrations of the different antagonistic filtrates exceeded 10%, AFB1 production was inhibited completely compared with that of the control.

In vivo studies on the effect of the selected actinobacterium isolate on A. flavus sporulation and AFB1 production in wheat

The number of *A. flavus* spores decreased to 92.3%, 99.6%, and 100% in treatment nos. 2, 3, and 4, respectively, where isolate no. 1 was inoculated after *A. flavus* was incubated for 7, 14, and 21 days, respectively. The AFB1 concentrations in wheat were reduced to 49.3% and 80.9% in treatment nos. 2 and 3, respectively. No aflatoxin was detected in treatment no. 4 compared with that in the control (Table 3).

AFB1 degradation by cell-free extracts

The AFB1 degradation by the cell-free extracts of the experimental isolate at different incubation periods was studied. The AFB1 concentration was significantly reduced when the incubation time was prolonged. After 4 days, it decreased by 95.4% (Table 4).

Effect of temperature on AFB1 degradation by the cell-free culture filtrate of the selected actinobacterium isolate

AFB1 degradation varied under different incubation temperatures. It increased as temperature increased until the optimum temperature was reached. Above the optimum temperature, the degradation rate began to decrease (Table 5). The AFB1 degradation was low (32.47%) at 40°C, but it was high (95.47%) at the optimum temperature of 30°C.

Cytotoxicity test of AFB1 degradation products

The cytotoxic effect of AFB1 and its degraded products was evaluated against brine shrimp (Table 6). The mortality rate of brine shrimp nauplii increased as the AFB1 concentration increased. Furthermore, 100% mortality was observed in the presence of 5µg/mL pure AFB1, but the AFB1 degradation products were slightly toxic.

Effect of shelf life and high temperature on antifungal activity

The comparison of the heat-treated cell-free culture filtrate with the fresh supernatant revealed that the antifungal activity of the cell-free culture filtrate decreased as temperature increased (Fig. 1). After being autoclaved at 121°C for 15min, the cell-free culture filtrate lost its antifungal activity (Table 7). Conversely, the antifungal substance remained highly stable for 12 months at freezing temperature, and the supernatant retained its antifungal activity (Table 8 and Fig. 2).

TABLE 1. Antagonistic activity of isolated actinobacteria against *Aspergillus flavus* using agar well diffusion method

Actinomycete isolate	Diameter of inhibition zone (mm)			
	Days			
	2	4	6	8
1	30.00 ± 0.00	28.00 ± 0.00	27.67 ± 0.58	27.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are mean ± standard deviation (SD).

TABLE 2. Effect of different concentrations of cell-free culture filtrate of the selected actinobacterium on *Aspergillus flavus*

CCF conc. (% v/v)	Dry weight (g/L) (Mean ± SD)	AFB_1 (µg/g) (Mean ± SD)	% of inhibition of dry weight	% of inhibition of AFB_1
Control	8.61 ± 0.48	465.82 ± 26.04	0.00	0.00
5	7.38 ± 0.25	407.08 ± 13.41	14.3	12.6
10	6.87 ± 0.60	73.12 ± 6.52	20.2	84.3
20	2.54 ± 0.21	0.00 ± 0.00	70.5	100
40	1.37 ± 0.28	0.00 ± 0.00	84.1	100
60	1.13 ± 0.16	0.00 ± 0.00	86.9	100
80	0.83 ± 0.13	0.00 ± 0.00	90.4	100
100	0.49 ± 0.10	0.00 ± 0.00	94.3	100

TABLE 3. Effect of the selected actinobacterium isolate on the numbers of spores of *Aspergillus flavus* and aflatoxin production in wheat after various incubation periods

Incubation periods (days)	(No. of spores/g) × 10^9	AFB_1 (µg/kg)
0	2.46 ± 0.055	1048.61 ± 3.36
1	0.24 ± 0.007	997.59 ± 2.44
2	0.19 ± 0.006	532.08 ± 1.31
3	0.01 ± 0.0004	199.69 ± 0.55
4	0.00 ± 000	0.00 ± 0.00

TABLE 4. Effect of cell-free culture filtrate of the selected actinobacterium isolate on the degradation of AFB_1 at different incubation periods

Incubation periods (days)	AFB_1 concentration (µg/mL)	% of degradation of AFB_1
0	5.00	0.0
1	1.15 ± 0.015	77.0
2	0.76 ± 0.012	84.8
3	0.32 ± 0.006	93.6
4	0.23 ± 0.006	95.4

TABLE 5. Effect of temperature on the degradation of AFB_1 by cell-free culture filtrate of the selected actinobacterium isolate

Temerature (°C)	AFB_1 concentration (µg/mL)	% of degradation of AFB_1
20	1.32 ± 0.006	73.6
25	1.33 ± 0.012	73.4
30	0.23 ± 0.006	95.4
35	0.27 ± 0.02	94.6
40	3.38 ± 0.012	32.4

TABLE 6. Effect of aflatoxin B_1 degradation products at different time intervals on mortality percentage of brine shrimp larvae

Days	Number of dead nauplii	% mortality
Control (AFB_1)	**10**	**100**
1	6.00 ± 0.00	60.00
2	5.67 ± 1.15	56.6
3	2.67 ± 0.58	26.7
4	3.00 ± 0.00	30

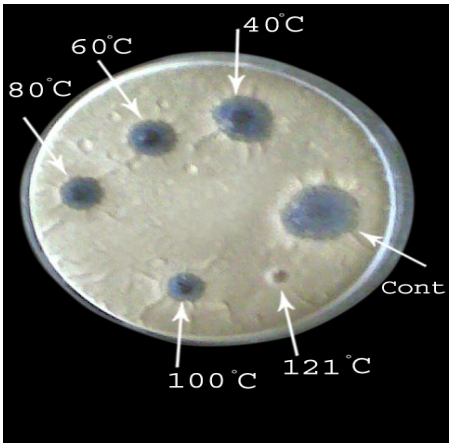

Fig. 1. Effect of high temperature on the antifungal activity of cell-free culture filtrate

TABLE 7. Effect of high temperature on inhibitory activity of cell-free culture filtrate against *Aspergillus flavus*

Temperature (ºC)	Inhibition zone (mm)
Control (30)	27.33 ± 0.58
40	11.67 ± 0.58
60	10.00 ± 0.00
80	7.33 ± 1.15
100	5.00 ± 0.00
121	0.00 ± 0.00

TABLE 8. Effect of shelf-life on the antifungal activity (inhibition zone) of cell-free culture filtrate

Incubation period (months)	Inhibition zone (mm)		
	Room temperature*	4ºC	Freezing temperature (-20ºC)
2	10.33 ± 0.58	27.67 ± 0.58	28.33 ± 0.58
4	0.00 ± 0.00	27.33 ± 0.58	28.00 ± 0.00
6	0.00 ± 0.00	19.33 ± 0.58	28.33 ± 0.58
8	0.00 ± 0.00	10.33 ± 0.58	27.67 ± 0.58
10	0.00 ± 0.00	0.00 ± 0.00	27.33 ± 0.58
12	0.00 ± 0.00	0.00 ± 0.00	27.33 ± 0.58

*Control (fresh cell-free culture filtrate) with inhibition zone of 27.33 ± 0.58mm.

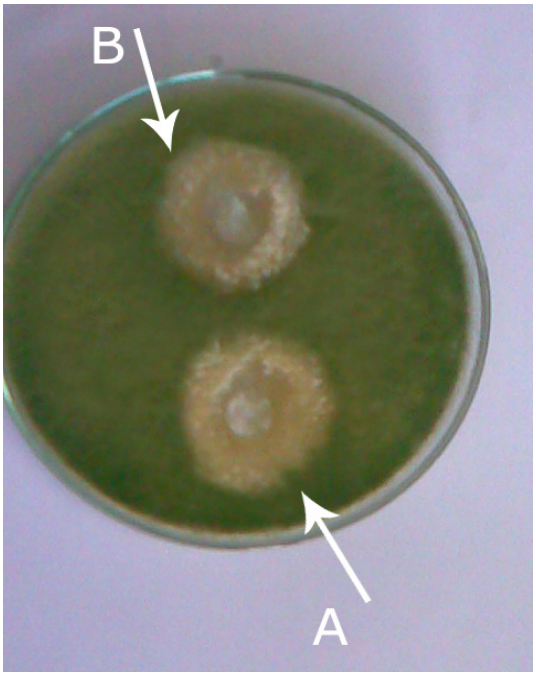

Fig. 2. Effect of 12-month freezing on the antifungal activity of cell-free culture filtrate (CCF), (A): Fresh CCF, (B): Frozen CCF

Characterization and identification of actinobacterium isolate no. 1

Among the four actinobacterium isolates, isolate no. 1 was the most active. It inhibited *A. flavus* growth and AFB1 production. As such, this isolate was further characterized and identified. The color of its aerial mycelia, the presence of LL-DAP in the cell wall, spore form, and physiological and biochemical characteristics (Figs. 3–5 and Tables 9–12) suggested that isolate no. 1 belonged to *Streptomyces*. The comparison of the characteristics of the isolate with those of the closest reference strains indicated that the isolate could be *S. exfoliatus*. Thus, it could be named *S. exfoliatus*. The characteristics of the selected isolate were also compared with those of the standard *S. exfoliatus* strain (ATCC 12627). The standard strain is characterized by rectiflexibiles spore chains, a smooth spore surface, spore mass in the gray series, and a wide variety of pigments responsible for the color of the vegetative and aerial mycelia. These characteristics were consistent with those of the isolate selected in this study.

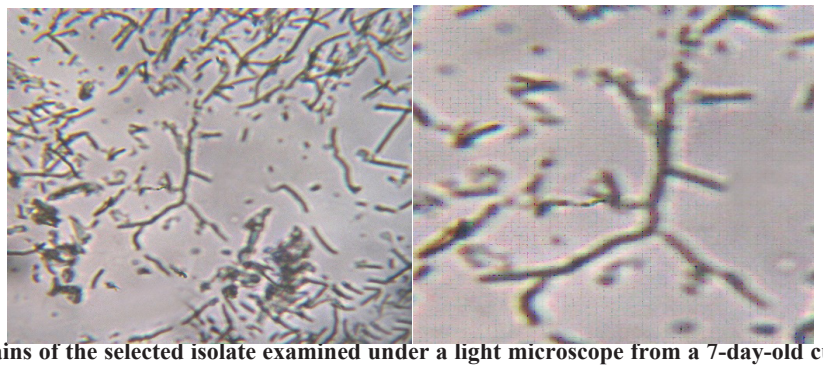

Fig. 3. Spore chains of the selected isolate examined under a light microscope from a 7-day-old culture on starch nitrate agar (400×)

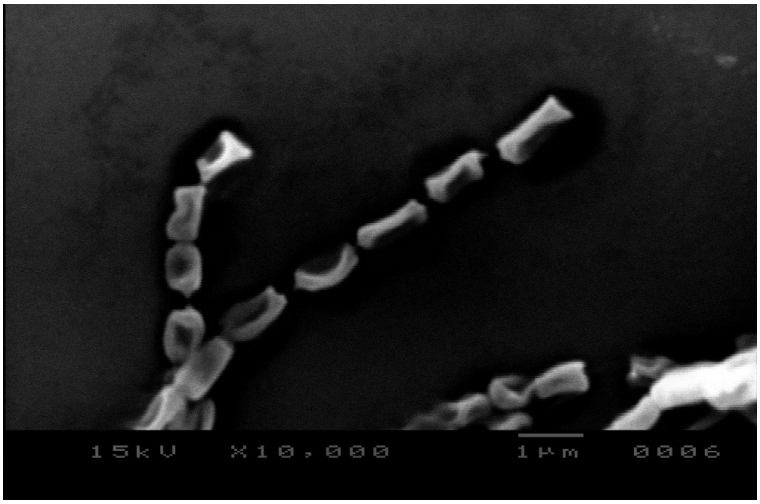

Fig. 4. Spore view of the selected isolate under a scanning electron microscope from a 14-day-old culture on starch nitrate agar (10,000×)

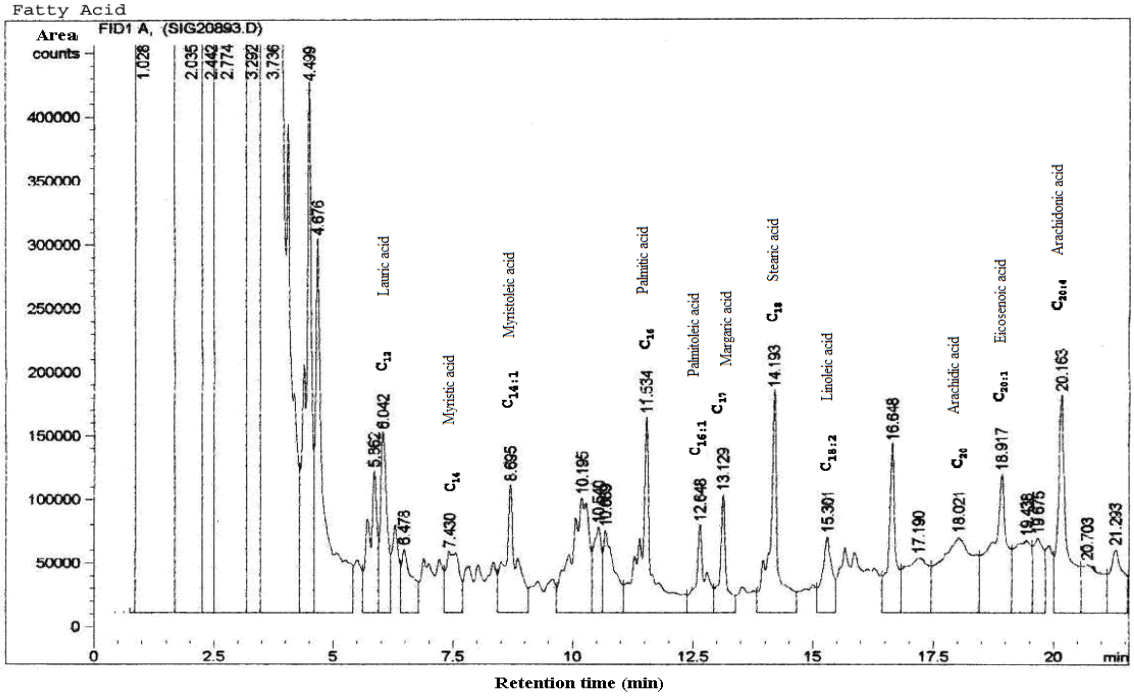

Fig. 5. Gas chromatography of fatty acid methyl esters of the selected actinobacterium isolate (isolate no. 1)

TABLE 9. Cultural characteristics of the actinobacterium isolate grown on different agar media for 14 days at 30°C

Media used	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar	Good	White to pinkish gray	Dark yellow	Yellowish brown
Inorganic salt starch agar	Poor	White	Grayish brown	Pale brown
Oatmeal agar	Good	White to grayish white	Colorless	None
Tyrosine agar	Moderate	None	Grayish olive	Pale yellow
Glycerol-asparagine agar	Good	Grayish white	Grayish brown	None
Glucose-asparagine agar	Poor	White	Pale brown	Yellowish brown
Starch nitrate agar	Very good	Grayish olive	Brown	Pale brown
Cazapek's-Dox agar	Moderate	Cream	Pale brown	None
Nutrient agar	Moderate	Grayish olive	Yellowish brown	None
Bennett's agar	Good	Beige	Pale brown	Yellowish brown
Sucrose nitrate agar	Moderate	Pinkish gray	Reddish brown	None

TABLE 10. Physiological characteristics of the selected actinobacterium isolate no. 1.

Test	Reaction	Test	Reaction
Melanin pigment	− ve	Temperature range for growth	20°C–45°C
Nitrate reduction	+ ve	NaCl-tolerance (3, 5, 7, 10%w/v)	3%–10%
H_2S production	+ ve	Sensitivity growth inhibitors:	
Gelatin hydrolysis	+ ve	a-Sodium-azide 0.01%	+ ve
Starch hydrolysis	+ ve	b-Phenol 0.1	+ ve
Xanthine degradation	+ ve	Resistance to antibiotics:	
Elastin degradation	+ ve	a-Rifampicin (50 µg ml^{-1})	− ve
L-Tyrosin degradation	+ ve	b-Penicillin G (10 i.u.)	+ ve
Allantoin degradation	+ ve	c-Garamycin (50 µg ml^{-1})	− ve
Tween 80 degradation	+ ve		
Casein degradation	+ ve	d-Streptomycin (50 µg ml^{-1})	− ve
Arbutin degradation	+ ve	e-Neomycin (50 µg ml^{-1})	− ve
Cellulose degradation	+ ve	Urease hydrolysis	+ ve
pH range for growth	6.5–8	Pectin hydrolysis	− ve
Antibiosis:			
E. coli	+ ve	Lipolysis	+ve
B. subtilis	+ ve	Proteolysis	+ve
A. niger	− ve	Lecithinase	+ ve
C. albicans	+ ve		

+ ve, positive; − ve, negative; i.u, international units.

TABLE 11. Biochemical characteristics of the selected actinobacterium isolate no. 1

Tested material	Reaction
Cell wall chemotype	I (LL-Diaminopimelic acid).
Whole cell sugar pattern	No diagnostic sugar.
Phospholipids	
PE (phosphatidylethanolamine)	+
Pl (phosphatidylinositol)	−
PIM (phosphatidylinositolmonoside)	−
DPG (diphoshatidylgycerol)	−
PG (phosphatidylglycerol)	−
PS (phosphaditylserine	−
Fatty acids pattern.	
Dodecanoic acid (lauric acid) (C12:0)	2.7%
Tetradecanoic acid (myristic acid) (C14:0)	18.0%
Tetradecenoic acid (myristoleic acid) (C14:1)	3.2%
Hexadecanoic acid (palmitic acid) (C16:0)	5.0%
Hexadecenoic acid (palmitoleic acid) (C16: l)	18.7%
Heptadecanoic acid (margaric acid) (C17:0)	16.6%
Octadecanoic acid (stearic acid) (C18:0)	4.1%
Octadecadienoic acid (linoleic acid) (C18:2)	16.8%
Eicosanoic acid (arachidic acid) (C20:0)	5.6%
Eicosenoic acid (eicosenoic acid) (C20:1)	4.8%
Eicosatetraenoic acid (arachidonic acid) (C20:4)	4.5%

(+), detected; (−), not detected.

TABLE 12. Utilization of different carbon and nitrogen sources by the selected actinobacterium isolate no. 1

Source	Utilization
D- Glucose	+
D- Xylose	+
Sucrose	−
L- Arabinose	+
L-Rhmonose	−
raffinose	−
Glycerol	+
Soluble starch	+
Cellulose	−
Mannitol	−
D-Fructose	+
Inositol	−
D-Galactose	+
Cellobiose	+
Maltose	+
K No_3	+
L- Phenyl alanine	+
L- Valine	−
L-Histidine	+
L-Arginine	+
L-Cystine	+

+, positive; −, negative.

Discussion

The biological control of *A. flavus* growth and aflatoxin production using antagonistic actinobacteria was the main target. As such, 80 isolates of actinobacteria were obtained from different soils in various governorates in Egypt, purified, and screened in terms of their ability to inhibit the growth of aflatoxigenic *A. flavus* via disc agar diffusion to achieve this control. The ability of these isolates to antagonize *A. flavus* considerably varied. The screening program using liquid cultures showed that isolate no. 1 was the most potent organism that exhibited the highest antifungal activity, whereas the other isolates did not show any activity.

Growth on carbon and nitrogen source in synthetic media and other characters are useful in species determination. Williams et al. (1989) indicated that the selected potent isolate is likely *S. exfoliatus*. Similarly, Ayari et al. (2012) reported that *Streptomyces* sp. S72 is a good producer of a compound with a moderate antifungal activity against *Aspergillus fumigatus* and *C. albicans* but without activity against *A. flavus*, *A. niger*, *C. pseudotropicalis*, and *C. tropicalis*. El-Sabbagh et al. (2013) found that *S. exfoliatus* is biologically active against *A. flavus*, *A. niger*, *F. oxysporum*, and *C. albicans*. In our study, the selected isolate was further characterized and identified. The results revealed that the selected isolate could grow well on the starch nitrate medium, Bennett's agar medium, and the glycerol–asparagine agar medium. However, it poorly grew on inorganic salt starch agar and glucose–asparagine agar media. It moderately grew on the other tested media. Furthermore, it was aerobic, spore forming, gram positive, and nonacid fast. It was isolated from soil, and its spore mass was gray. Its aerial mycelium had long straight filaments. LL-DAP is present in the cell wall. Phospholipids were PII in the complete cell, with no diagnostic sugar. The major fatty acids were tetradecanoic acid (14:0) and hexadecanoic acid (16:0) based on fatty acid patterns. Its DNA had 71.0 mol% G + C. All the previous characters confirmed that the isolate belonged to *Streptomyces*. For species identification, microscopic examination showed a smooth spore surface and a rectiflexibile spore chain. This isolate could grow in the presence of sodium azide (0.01% w/v), phenol (0.1% w/v), and sodium chloride (3%, 5%, 7%, and 10% w/v), but it was susceptible to penicillin G (10 i.u). It could decompose allantoin, arbutin, lecithin, casein, Tween 80, tyrosin, elastin, xanthine, and starch. It could also hydrolyze gelatin, produce H_2S, and reduce nitrate. This isolate could inhibit the growth of *B. subtilis*, *E. coli*, and *C. albicans*.

As the concentrations of the cell-free culture filtrate of *S. exfoliatus* increased until 10%, *A. flavus* growth and AFB1 production gradually decreased. At >10% concentrations, aflatoxin production was completely inhibited compared with that of the control. The antifungal potential of the culture filtrate was probably related to the high production of secondary antifungal compounds as antibiotics in many *Streptomyces* species (Taechowisan et al., 2005; Hassan et al., 2011). Terrestrial actinobacteria, especially those classified under *Streptomyces*, are rich sources of antifungal and AF inhibitory metabolites (Yoshinari et al., 2007; Shakeel et al., 2018; Benkerroum, 2020). These findings suggested that the inhibition of fungal growth by culture filtrates was probably attributed to the production of extracellular hydrolytic enzymes and secondary antifungal compounds.

Our *in vivo* study revealed that the spore concentration and aflatoxin production decreased as time lapsed, and AFB1 production and *A. flavus* growth were completely inhibited in treatment no. 4. Waliyar et al. (2015) showed that isolates of *Trichoderma* spp. significantly decrease aflatoxin infection in groundnuts by 20%–90%. *B. subtilis* UTBSP1 can reduce *A. flavus* growth and AFB1 contamination in pistachio nuts (Farzaneh et al., 2016). Cuero et al. (1991) conducted field tests involving the bacterial inoculation of corn ears for 48h prior to *A. flavus* inoculation. They demonstrated that *B. subtilis* prevents aflatoxin contamination in corn. However, they observed that aflatoxin contamination is not reduced when they inoculated bacteria 48h after *A. flavus* inoculation. The cell-free extracts of *B. vallismortis* and *B. amyloliquefaciens* prevent *Aspergillus* sp. growth and AFB1 production in grape (El-Shanshoury et al., 2018).

The cell-free culture filtrate of *S. exfoliatus* showed a strong AFB1 degrading activity. It could degrade 95.4% of AFB1 after 72h of incubation. AFB1 was degraded by the cell-free culture filtrate produced without pre-exposure to AFB1, indicating that degradation occurred during the normal growth of the bacterium. This finding

suggested that this degradation was a constitutive activity of *S. exfoliatus*. Similar results were obtained in previous studies. For example, Alberts et al. (2006) reported a 66.8% reduction of AFB1 from 0 h to 72 h during incubation with the culture supernatant of *Rhodococcus erythropolis*. Garai et al. (2021) indicated that *R. erythropolis* NI1 strain can degrade mycotoxins and their mixtures in different proportions. They also found that the ratio of mycotoxins significantly reduced in combination is higher than that in single ones. Niu et al. (2008) demonstrated that AFB1 degradation by the culture supernatant of *S. maltophilia* 35-3 is a relatively rapid and continuous process. In this process, 46.3% AFB1 is degraded in the first 12h, and 78.7% is degraded after 72h. Farzaneh et al. (2012) observed that the cell-free supernatant fluid of *B. subtilis* strain UTBSP1 isolated from pistachio nuts decreases the AFB1 content by 78.39% after 24h.

The effect of different incubation temperatures on AFB1 degradation by the cell-free culture filtrate of *S. exfoliatus* was also evaluated. After an incubation time of 72 h at 20°C, 25°C, 30°C, 35°C, and 40°C, the residual AFB1 contents were determined. The result showed that optimum degradation occurred at 30°C. Similarly, Teniola et al. (2005) reported that AFB1 degradation by the cell extract of *R. erythropolis* is the same as that of *Mycobacterium fluoranthenivorans* between 10°C and 40°C (>90%). They also observed optimal degradation at 30°C. They proposed that enzymes in these extracts are active in a wide temperature range. The AFB1 degradation by *S. maltophilia* 35-3 culture supernatant varied under different temperatures. The degradation was lower at 20°C (60.8%) and 30°C (63.5%) than at 37°C (78.7%). Farzaneh et al. (2012) detected the optimal conditions for the AFB1 degradation by the cell-free supernatant of *B. subtilis* strain UTBSP1 at 35°C–40°C for 24h. The destroyed AFB1 chemically differed from the standard AFB1 and lost its fluorescence property. In this study, the antifungal metabolites of *S. exfoliatus* were subjected to different temperature ranges to determine the effect of temperature on the stability of the antifungal activity of the cell-free culture filtrate. Its antifungal activity decreased as temperature increased. However, its antifungal activity was completely lost after it was autoclaved at 121°C for 15min. The stability of the antifungal metabolites was the highest in the cell-free culture filtrates stored at freezing temperature, followed by those stored at 4°C. The least stable were the cell-free culture filtrates stored at room temperature. Although the activity of the antifungal metabolite was recorded during the entire test, the antifungal activity decreased as the duration was prolonged. Similarly, previous studies showed that the antimicrobial activity of antibiotic metabolites exposed to different temperatures decreases (Augustine et al., 2005; Oskay, 2009).

Brine shrimp larvae are convenient test organisms for bioassay toxic fungal metabolites, such as mycotoxins (Moretti et al., 2007). Duraković et al. (2011) and Mohammed & Al-Ani (2021) observed that brine shrimp toxicity is positively correlated with mycotoxins. In the current study, the bioactivity of the degraded AFB1 product against *A. salina* was determined using methanol as a solvent, which is the least toxic organic solvent (Price et al., 1974). The degree of lethality was directly proportional to the concentration of AFB1. The maximum mortality (100% mortality) was detected at the control concentration (5µg/mL), whereas the least mortality (26.7%) was observed at 0.23µg/mL. The results of the bioassay with AFB1 and AFB1 degraded product confirmed that a novel substance was formed due to bacterial degradation of AFB1. It was less toxic than the parent compound (AFB1).

Conclusion

In conclusion, the *S. exfoliatus* strain and its cell-free culture filtrate may be promising options for lowering *A. flavus* contamination since they prevent aflatoxin formation, enhance aflatoxin breakdown, and induce AFB1 detoxification. In future biological control endeavors, this approach could be employed to reduce *A. flavus* and AFB1 contamination. Thus, the selected *S. exfoliatus* strain and its cell-free culture filtrate could be candidates for AFB1 detoxification.

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Reviewing; H.E.S: Methodology, investigation and writing original draft.

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المقاومه الحيوية للأفلاتوكسين ب 1 بواسطة الإستربتوميسيس إكسفولياتس

عبد الرحيم رمضان الشنشورى[1]، متولي عبد العظيم متولى [1]، صابحه محمود الصباغ[2]، حمدى على عماره [3] ، هبة الله المرسي سبع[1]

[1] شعبة الميكروبيولوجى - قسم النبات والميكروبيولوجى- كلية العلوم - جامعة طنطا - طنطا - مصر، [2]قسم النبات-كلية العلوم - جامعة المنوفيه - شبين الكوم - مصر، [3]قسم الميكروبيولوجى - معهد بحوث الأراضى والمياه -- مركز البحوث الزراعية - جيزه - مصر.

في المناطق الاستوائية وشبه الاستوائية، يعد تلوث المحاصيل بالأفلاتوكسين تحديًا متزايدًا. للحد من نمو الفطريات وإزالة السموم من الأفلاتوكسين، هناك حاجة لبذل جهود للحد من تلوث السموم الفطرية. أظهرت البكتيره استربتوميسيس إكسفولياتس نتائج واعدة ضد العديد من الفطريات الممرضة للنبات. هدفت الدراسة الحالية إلى استكشاف ما إذا كان من الممكن استخدام تلك البكتيره كعامل تحكم بيولوجي ضد فطرالأسبرجلس فلافس والتلوث بالأفلاتوكسين. تمت فى هذه الدراسة المكافحة الحيويه للفطره أسبرجلس فلافس المنتجه للأفلاتوكسين ب 1 بإستخدام بعض عزلات البكتيريا الشعاعيه كانت عزلة الأكتينوبكتيريا رقم 1 والتي تم تعريفها بإسم إستربتوميسيس إكسفولياتس هي الأكثر فعالية ضد فطرالأسبرجلس فلافس. تمت دراسة تأثير الراشح المزرعي الخالى من الخلايا لسلالة الإستربتوميسيس إكسفولياتس على نشاط الفطر حيث قام بتثبيط كل من نمو الفطر وإنتاجه للجراثيم وللسم الفطري الأفلاتوكسين ب 1. وتم تثبيط إنتاج الأفلاتوكسين كلية عند تركيز 20% (حجم/حجم) للراشح المزرعي. وكان تأثير حقن السلالة التضادية الإستربتوميسيس إكسفولياتس فى حبوب القمح أدى الى خفض تركيز الأفلاتوكسين ب 1 في عينات القمح كلما زاد الوقت وكانت أفضل معامله لحقن السلاله التضاديه هي قبل حقن الفطر بحوالى 21 يوم، بالمقارنة مع حقن الفطر والسلاله التضادية فى نفس الوقت حيث تم تثبيط إنتاج الأفلاتوكسين ب 1 تماما. وقد كان للراشح المزرعي لسلالة الإستربتوميسيس إكسفولياتس القدره على تحلل الأفلاتوكسين ب 1 بفاعلية 95.47% عند درجة حرارة 30 درجة مئويه بعد 3 أيام والذي انخفض بزيادة درجة الحراره. تم إختبار النشاط السام للمنتجات المتحللة من سم الأفلاتوكسين بإستخدام يرقات بيض الجمبرى، حيث أوضحت النتائج أن تلك النواتج المتحلله تقل سميتها نتيجة المعاملة بالراشح المزرعى بالمقارنه مع الأفلاتوكسين الخام. وقد وجد أيضا أن تأثير الراشح المزرعى المتجمد لسلالة الإستربتوميسيس إكسفولياتس لا يتغير حتى 12 شهر. في الخلاصه، يمكن لفلترات المستنبتات الخالية من الخلايا للبكتيره قيد الدراسة أن تمنع نمو فطرالأسبرجلس فلافس وتكوُّن الأبواغ، بالإضافة إلى تقليل توليد الأفلاتوكسين ب 1 وتحطيمه إلى مركبات أقل ضررًا. يبدو أن النتائج المهمة لتقليل فطرالأسبرجلس فلافس، وكذلك تحلل وتراكم الأفلاتوكسين في حبوب القمح، واعدة وتقترح أنه يمكن استخدام هذا النهج لتقليل تلوث فطرالأسبرجلس فلافس والأفلاتوكسين في برامج المكافحة البيولوجية المستقبلية.