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AN EFFECTIVE AFLATOXIN B_1 REDUCTION IN WHEAT GRAIN CONTAMINATED BY *Aspergillus flavus* VIA COMBINING THE BIOLOGICAL DEGRADATION OF THE TOXIN WITH INHIBITION OF ITS BIOSYNTHESIS

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Abstract

Decontamination of forage grain polluted with mycotoxins is one of the relevant problems of the forage safety provision. In recent years, the frequency of a severe contamination of forage grain and other fodder with aflatoxin B_1 (AFB1) in Russia significantly increased. The probability of the AFB1 contamination of the grass stand and forage grain produced in the central and northern regions of Russia may increase in the future due to the further expansion of *Aspergillus flavus* fungus, the main AFB1 producer, into these regions as a result of climate change. One of the promising approaches to decontaminate grain contaminated with AFB1 is the toxin catabolization by various microorganisms producing enzymes able to degrade AFB1. Another approach includes the treatment of grain contaminated with the AFB1 producers with compounds able to inhibit the aflatoxigenesis. In the present work, it was shown for the first time that the treatment of the cultural broth of *Rhodococcus erythropolus* AC-884 with the supernatant after the treatment with compactin almost completely prevents the accumulation of mycotoxin in the infected grain. The aim of the work is to evaluate the effectiveness of reducing the content of mycotoxin in wheat grain artificially contaminated with aflatoxin B_1 after treatment with actinobacteria of the genus *Rhodococcus* or an inhibitor of aflatoxygenesis, the compactin, as well as a combination of these methods. The present study reports the results of investigation of the AFB1 destruction capability in four *Rhodococcus* strains (*Rhodococcus* sp., AC-1260, *R. erythropolus* AC-1269 and AC-884, and *R. ruber* AC-1801). Quantitative analysis of AFB1 by high performance liquid chromatography revealed that the most active mycotoxin degradation occurred in the cell-free cultural broth supernatant of AC-884 (CBS-884). Only trace amounts of AFB1 added in CBS-17 to a final concentration of 0.2 μg/ml were detected in this supernatant after 48-h incubation at 30 °C, whereas cultural broth supernatants of other studied strains contained from 15 to 50 % of the added AFB1 after its incubation under the same conditions. A 72-h treatment of wheat (*Triticum aestivum* L., cv. Daria) grain artificially contaminated with AFB1 (1.0, 2.5, or 5.0 μg/g) with CBS-884 removed 60 % of the toxin, while the use of cultural broth supernatants or cell suspensions of AC-1260, AC-1269 or AC-1801 strains did not result in any changes in the AFB1 content comparing to the control. Using a consecutive treatment of grain infected with a toxigenic *Aspergillus flavus* strain by compactin, inhibiting the AFB1 production in this fungus, and then by CBS-884, we first demonstrated that the approach based on application of AFB1 biosynthesis inhibitors followed by the toxin biodegradation allowed an efficient decontamination of grain if the use of inhibitors alone did not result in a complete suppression of the aflatoxigenesis. Grain treatment of with the supernatant of AC-884 was more effective than the treatment with a similarly obtained supernatant of another AFB1-destroiyng agent, *Phoma glomerata* PG-41. In our experiments, the AFB1 content in wheat grain contaminated with *A. flavus* A11 reduced twice compared to the control in 7 days after compactin (0.05 mg/g) application. In 24 h after the treatment of the contaminated grain with CBS-884 alone (0.25 ml/g), the AFB1 amount produced by *A. flavus* for six post-inoculation days was reduced almost thrice. Combination of both treatments (compactin, 0.05 mg/g, and then CBS-884, 0.25 ml/g) resulted in a more than 200-fold reduction of the AFB1 content and the achievement of almost complete grain

decontamination. Therefore, an approach based on a combination of biodegradation with inhibition of aflatoxigenesis can provide effective decontamination of grain contaminated with AFB1 producers in cases where the use of inhibitors does not lead to complete suppression of mycotoxin biosynthesis.

Keywords: aflatoxin B_1, forage, grain, decontamination, *Aspergillus flavus*, compactin, *Rhodococcus erythropolus*

In recent years, samples of feed grain, silage and other feed contaminated with mycotoxins [1-3] collected in Russia have increasingly been found to contain aflatoxin B_1 (APB_1) [4, 5], including in amounts exceeding its maximum allowable concentrations [2, 6, 7]. Since global warming promotes the migration of heat-loving micromycetes to zones with a temperate and cold climate, we should expect an expansion of the range of *Aspergillus flavus* and other APB_1-producing aspergillus species in these regions and, accordingly, an increase in the likelihood of aflatoxin contamination of grass stands and fodder grains of cereals cultivated there.

Like other mycotoxins, AFB_1 enters the food chain and can end up in food, posing a serious threat to human health due to high hepatotoxicity and hepatocarcinogenic activity [8]. Entering the animal body with food, AFB_1 is metabolized with the formation of DNA-damaging exo-8,9-epoxide and aflatoxin M1 [9, 10], which accumulates in poultry eggs, cow milk and, like AFB_1 itself, has carcinogenic, teratogenic, mutagenic and immunosuppressive effects [11].

Due to the high risk of AFB_1, the development of effective methods for reducing the contamination of feed grains used in various livestock industries is one of the most important tasks in ensuring safety of agricultural feed. To solve it, researchers involve both physical, chemical, and biological methods of decontamination [12, 13]. Among the latter, a special place is occupied by biodegradation, an approach based on the destruction or detoxifying transformation of AFB_1 by degrading microorganisms [14, 15] which secrete metabolites with the corresponding enzymatic activity [16-18].

The ability to biodegrade aflatoxins has been found in many microorganisms, including actinobacteria of the genus *Rhodococcus* [19-22]. Nevertheless, the study of the destructive potential of this taxonomic group toward FB1 in order to identify the most effective strains is still relevant, since the ability to produce aflatoxin-degrading metabolites is not species-specific. Both *Rhodococcus* species and strains within the same species differ significantly in productivity [20, 22]. In addition, almost all studies reported only the ability of these actinobacteria to degrade AFB_1 when added to the nutrient medium, and only in very rare cases, the possibility of using bacteria to degrade AFB_1 extracted from contaminated grain or grain processing waste [23].

The content of AFB_1 in grain contaminated with its producers can also be reduced with synthetic or biological inhibitors of aflatoxygenesis [12] treatment with which will prevent the accumulation of the toxin in the infected substrate. The promise of this approach was demonstrated by us in previous studies which showed that compactin, one of the secondary metabolites of *Penicillium citrinum*, is able to suppress the formation of AFB_1 in *Aspergillus flavus* [24]. However, with an increase in temperature and humidity in granaries, conditions are often created that are favorable for the rapid and intensive development of aspergillus, which can significantly reduce the effectiveness of inhibitors due to insufficiently complete suppression of toxigenesis. To ensure the necessary decontamination, toxin residues in inhibitor-treated grain can be eliminated through biodegradation.

In the present work, it was shown for the first time that the treatment of the culture liquid of *Rhodococcus erythropolus* AC-884 with the supernatant following the treatment with compactin almost completely prevents the accumulation of mycotoxin in the infected grain.

The purpose of this work is to evaluate the effectiveness of reducing the content of mycotoxin in wheat grain artificially contaminated with aflatoxin B_1 after treatment with actinobacteria of the genus *Rhodococcus*, an inhibitor of aflatoxygenesis (compactin), or their combination.

Materials and methods. Strains of *Rhodococcus* sp. AC-1260, *R. ruber* AC1801, *R. erythropolus* AC-1269, and AC-884 (All-Russian Collection of Industrial Microorganisms, State Research Institute of Genetics and Breeding of Industrial Microorganisms, Kurchatov Institute, Moscow) were cultured on R1 medium containing (g/l) peptone 15.0, yeast extract 6.0, NaCl 1.0, glucose 1.0; pH 7.0, which is used in testing bioactivity of actinobacteria of the genus *Rhodococcus* [25]. Equal amounts of bacterial inoculum (10^9 cells/ml) were introduced into 50 ml flasks with 10 ml of nutrient medium and grown for 48 h (an Excella® E-25R orbital shaker, New Brunswick, USA) at temperature of 30 °C and 290 rpm. Flasks with nutrient media without actinobacteria were kept under the same conditions (control). The bacterial biomass (number of cells/ml) was determined at the end of growth.

Toxin-degrading activity of metabolites of *Phoma glomerata* strain PG-41 (the State Collection of Phytopathogenic Microorganisms of the All-Russian Research Institute of Phytopathology), the AFB_1 biodestructor previously discovered by us [26] was compared with the activity of *R. erythropolus* AC-884.

To assess the ability of the above collection strains of actinobacteria to produce metabolites degrading AFB_1, bacterial cultures were centrifuged for 30 min at 10,000 g (5702 R, Eppendorf AG, Germany). Prior to toxin addition, the resulting supernatants were pH adjusted to 7.0 to prevent non-enzymatic reversible lactone ring opening in AFB_1, which occurs in an alkaline environment at high pH values [27], This can lead to false positive results in high performance liquid chromatography (HPLC) analysis. The supernatants were filtered through membranes (0.22 μm, Millipore, USA). AFB_1 (Sigma-Aldrich, USA) was added to the supernatants free from bacterial cells, as well as to the supernatants of control media to a concentration of 0.2 μg/ml, the mixture was incubated at 30 °C with stirring on a shaker for 24 h at the conditions indicated above, and the content of the toxin in the mixture was measured.

In experiments on the degradation of AFB_1 upon artificial contamination, grain of soft spring wheat (*Triticum aestivum* L.) cv. Darra, placed in 250 ml shaking flasks (20 g per flask), were soaked in water (10 ml per 20 g of grain) and autoclaved for 1 h at 1 atm. After sterilization, 1 ml of AFB_1 stock solutions in 20% ethanol were added to the flasks to final concentrations of 1.0; 2.5 and 5.0 μg/g of grain and vigorously shaken for 10-15 min to evenly distribute the toxin. Then 1 ml of 20% ethanol was added to the control flasks. *Rhodococcus* cultures were grown and centrifuged as described above, the supernatants were separated, and the pellets were suspended in 20 ml of sterile water. Concentration-equalized cell suspensions of the tested bacterial strains were added to the flasks with grain at the rate of 0.05 ml of suspension per 1 g of grain or 0.25 ml of supernatants. The same volumes of sterile water were added to the control. When comparing the toxin-degrading activity of the cell suspension and the *R. erythropolus* AC-884 culture liquid supernatant, the grain contaminated with the maximum concentration of AFB_1 (5.0 μg/g) was treated with supernatant.

In experiments with decontamination of grains infected with toxigenic *A. flavus*, flasks with sterile grains were infected with strain A11 (107 conidia/ml, 1 ml suspension/20 g grain) and divided into four batches. Compactin was added to the first batch to a final concentration of 0.05 mg/g. In the second batch, the grain, 6 days after inoculation with a conidial suspension of the same concentration, was treated with the supernatant of the culture liquid (CL) of strain AC-884

(0.25 ml/g). In the third batch, the grain was first treated with compactin (0.05 mg/g), and in 6 days, the above amount of supernatant was added. Control flasks were supplemented with 20% ethanol, sterile water, or both ethanol and water.

The flasks with grain artificially contaminated with the toxin were kept in a TSO-1/80 thermostat (OAO Smolenskoye SKTB SPU, Russia) at 30 °C for 3 days after the addition of biodestructors, and the flasks with contaminated grain were kept at 27 °C for 7 days after inoculation. Control flasks were incubated for 3 or 7 days under the same conditions.

Spores of toxigenic *A. flavus* A11 for wheat grain inoculation were obtained as previously described [24]. Compactin was obtained from *P. citrinum* (strain 18-12) CL using the method described by S.N. Ukraintseva et al. [28].

Cultures of the fungus *P. glomerata* PG-41 secreting AFB_1-degrading metabolites were grown in a liquid nutrient medium as described previously [26], and supernatants were obtained from the CL filtrate by centrifuging and sterilizing it in the same way as in experiments with bacterial cultures. *P. glomerata* supernatants were used in experiments on the degradation of AFB_1 and decontamination of grain infected with *A. flavus* A11, similarly to the protocol described above for bacterial strains.

Residual amounts of APB_1 extracted from supernatants of CL and wheat grain by extraction with chloroform were mwasured by high performance liquid chromatography (Waters 1525 Breeze HPLC SYSTEM, Waters Corp., USA) [26, 28]. The limit of detection of AFV_1 was 0.005 μg/g of grain (maximum concentration limit in grain was 0.01-0.5 μg/g), the completeness of extraction from CL was at least 80%.

Experiments (at least 3 repetitions per option in each) were repeated three times. Statistical processing was performed using the STATISTICA 6.1 program (StatSoft, Inc., USA). Mean (M) and standard deviation (±SD) values were calculated. Significance of differences between the control and experimental variants at $p \leq 0.05$ was determined using a t-test for independent variables.

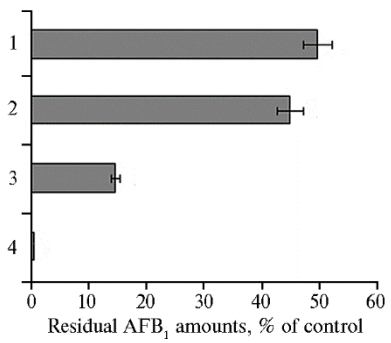

Fig. 1. The ability of various strains of *Rho*dococcus spp. to reduce the content of aflatoxin B_1 (AFB_1) added to the supernatant of their culture liquid. Average values for three experiments: 1 — *R. ruber* AC1801, 2 — *Rhodococcus* sp. AC-1260, 3 — *R. erythropolus* AC-1269, 4 — *R. erythropolus* AC-884 (n = 9, M±SD).

Results. All *Rhodococcus* strains actively grew on the R1 medium. Their biomass on day 2 reached 10^{10} bacterial cells/ml. By the end of the test period, pH values of CL did not differ among the strains and ranged from 8.8 to 9.0. These results indicate that the potential AFB_1 biodegraders in our experiments were cultured under conditions that provided a correct comparative assessment of their ability to produce metabolites with the target activity and could not prevent the realization of this ability in a particular strain.

Analysis of the residual amounts of AFB_1 after its incubation in CL supernatants showed that among the tested *Rhodococcus* cultures, the *R. erythropolus* AC-884 was the most active producer of toxin-degrading metabolites. After incubation of AFB_1 in the CL supernatant of this strain at neutral pH for 24 h, only trace amounts of the toxin were detected (Fig. 1). Under the same conditions, the CL supernatants of three other strains retained from 15 to 50% of the added AFB_1 (see Fig. 1).

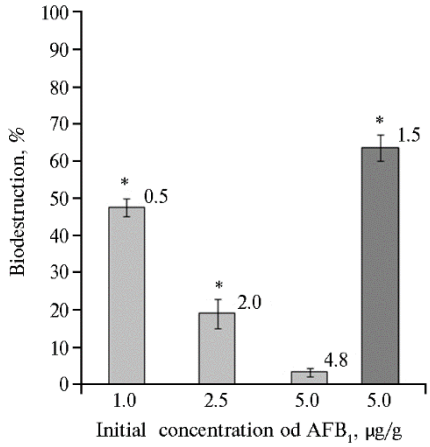

Fig. 2. Efficiency of degradation of aflatoxin B_1 (APB_1) added to grain of wheat (*Triticum aestivum* L.) cv. Daria, using a cell suspension (light gray columns) **or supernatant of the culture liquid** (dark gray column) **of *Rhodococcus erythropolus* AC-884, depending on the initial concentration of the toxin** (n = 9, M±SD). The numbers next to the columns show the residual amounts of AFB_1 in the grain (μg/g) 3 days after treatment. *Differences from control are statistically significant at $p \leq 0.05$ (marked with an asterisk).

In the analysis of grain artificially contaminated with commercial AFB_1, a statistically significant decrease in its content compared to the control was found (at $p \leq 0.05$)only if the treatment was carried out using strain AC-884. Three days after the introduction of the bacterial suspension, the amount of AFB_1 in the grain decreased by almost 2 times compared to the control (degradation efficiency 47.6%) if the final concentration of the toxin was 1.0 rg/g grain. Statistically significant differences with the control after treatment with the cell suspension ($p = 0.003$) remained at the AFB_1 concentration of 2.5 μg/g.

The treatment of grain contaminated with the maximum dose of the toxin (5.0 μg/g) with CL AC-884 supernatant led to the removal of 60 to 70% of AFV_1 within 3 days (Fig. 2). In addition, the degradation of AFB_1 in the CL supernatant of *R. erythropolus* AC-884 occurred faster than in the similarly obtained extracellular supernatant of the culture of the fungus *P. glomerata* PG-41, another AFB_1 biodegrader that actively removed the toxin from model incubation media [26]. However, unlike the supernatant, CL AC-884 proved to be ineffective when used on contaminated grain (Fig. 3).

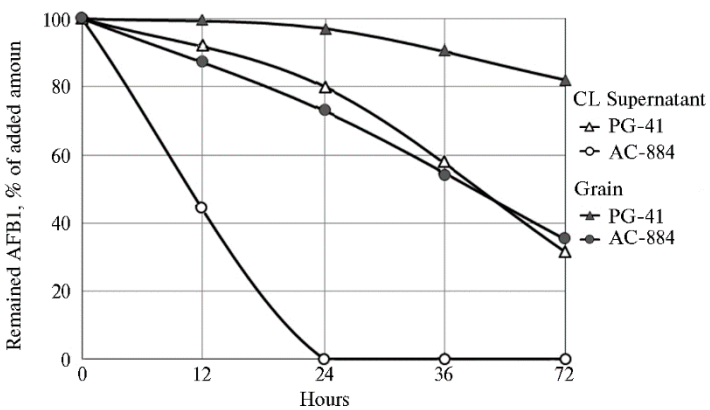

FIg. 3. Dynamics of degradation of aflatoxin B_1 (AFB_1) for 3 days in the supernatant of the culture liquid of *Rhodococcus erythropolus* AC-884 or *Phoma glomerata* PG41 and in treated wheat (*Triticum aestivum* L.) cv. Daria grain artificially contaminated with the toxin.

The high toxin-degrading activity of metabolites secreted by strain AC-884 in CL was confirmed by experiments with infection of wheat grains with AFB_1-producing *A. flavus* strain (Table). One day after treatment with the supernatant of grain samples on which the fungus developed for 6 days, the toxin content in the extracts obtained from these samples decreased by almost 3 times compared to the control, and the use of an inhibitor led to a 5-fold decrease in contamination.

Accumulation of aflatoxin B_1 (AFB_1) in *Aspergillus flavus* A11-infected grain of wheat (*Triticum aestivum* L.) cv. Daria after separate or combined treatment of grain with compactin and metabolites of *Rhodococcus erythropolus* AC-884 ($n = 9$, $M \pm SD$)

Treatment	AFB_1, μg/ml	Decontamination, %
Compactin, 0.05 mg/g grain	7.45±1.34	78.5
Culture liquid supernatant (CL) AC-884, 0.25 ml/g grain	12.10±1.27	65.1
Compactin, 0.05 mg + CL supernatant AC-884, 0.25 ml/g grain	0.13±0.09	99.6
Control (no processing)	34.66±3.07	

Note. Differences from control and between treatments are statistically significant at $p < 0.05$.

As a result of the successive application of compactin and supernatant CL AC-884, the concentration of AFB_1 in the extracts decreased by more than 200 times (see Table), that is, the combination of two treatments provided almost 100% grain decontamination. Since the supernatant freed from bacterial cells was used in these experiments, the decrease in the content of AFB_1 in the grain is due to the action of metabolites secreted by bacteria in the CL, and not to the often occurring adsorption of the toxin by biodestructor cells [29] that can be reversible [30] and led to the fact that this decontamination method does not always retain its effectiveness [31].

The results of our previous studies [26], the sensitivity of the detected AFB1-degrading activity to proteolysis, and the analysis of literature data suggest that the activity of AC-884 supernathans is of an enzymatic nature. Thus, enzymes that catalyze the degradation of fungal toxins, for example, ergot alkaloids (ErgA hydrolase, ErgB amidase) [32], or are involved in the catabolism of toxic polyaromatic compounds (for example, biphenyl dioxygenase and dehydrogenase), have been identified in a number of *R. erythropolus* strains. It has been established that the genes encoding these enzymes are grouped into clusters, and enzymatic degradation occurs through a cascade of reactions, including the cleavage of aromatic rings. Since APB_1 is also a polyaromatic compound, it is hypothesized that it may be targeted by similar enzymes and be degraded in a similar way [33].

The high decontaminating potential of AC-884 CL supernatant in our experiments is consistent with previously obtained data on the ability of extracellular supernatants of other *R. erythropolus* shams to degrade AFB_1, while significantly reducing its genotoxicity [33]. Apparently, the synthesis of AFB_1-degrading enzymes is a constitutive property of many *R. erythropolus* strains, although, unlike AC-884, not all of them are able to secrete such CL enzymes [22].

In our studies, the use of the AFB_1 biosynthesis inhibitor together with the subsequent removal of residual amounts of toxin through their biodegradation led to almost complete decontamination of contaminated grain. The possibility of practical implementation of this approach will be tested in further experiments by processing samples of stored grain collected in areas at risk of contamination by aflatoxin producers and contaminated silage. It is also very promising that some strains of *R. erythropolus* are able to degrade mycotoxins T-2 [20] and zearalenone [18], as well as to use ochratoxin A as a source of phenylalanine [34]. Since we have recently discovered that a compactin analogue can inhibit the formation of zearalenone [35], it seems reasonable to test the ability of AC-884 to degrade this fusariotoxin and other mycotoxins. If such an ability is discovered, the scope of the developed approach can be significantly expanded. The availability of sources of target metabolites, namely, the aflatoxin-degrading activity in the CL supernatant of *R. erythropolus* AC-884 and the ability of *P. citrinum* 18-12 to secrete compactin in vitro [26], is a factor that in the future may contribute to the development of a relatively simple technology for a two-component biological product

for decontamination of feed grains and other feeds.

Thus, as a result of the study of the toxin-degrading ability of four strains of *Rhodococcus* (*Rhodococcus* sp. AC-1260, *R. ruber* AC1801, *R. erythropolus* AC-1269 and *R. erythropolus* AC-884) and its comparison in experiments with grain treatment with culture liquid supernatant (CL) of *Phoma glomerata* PG-41, the most active biodegrader of aflatoxin B_1 (AFB_1), *R. erythropolus* AC-884, was selected. The efficiency of decontamination of grain artificially contaminated with a toxin preparation after treatment with AC-884 culture liquid supernatant could reach 70%, and after application on grain infected with toxigenic *Aspergillus flavus*, it averaged 65%. The treatment of such grain with compactin reduced the content of AFB_1 with an efficiency of almost 79%. Combined treatment, i.e., addition of supernatant CL AC-884 after compactin, actually completely prevented the accumulation of mycotoxin in wheat grain infected with *A. flavusinfected*.

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