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Aflatoxin B_1 inhibition in *Aspergillus flavus* by *Aspergillus niger* through down-regulating expression of major biosynthetic genes and AFB_1 degradation by atoxigenic *A. flavus*

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ABSTRACT

Twenty *Aspergillus niger* strains were isolated from peanuts and 14 strains were able to completely inhibit AFB_1 production with co-cultivation. By using a Spin-X centrifuge system, it was confirmed that there are some soluble signal molecules or antibiotics involved in the inhibition by *A. niger*, although they are absent during the initial 24 h of *A. flavus* growth when it is sensitive to inhibition. In *A. flavus*, 19 of 20 aflatoxin biosynthetic genes were down-regulated by *A. niger*. Importantly, the expression of *aflS* was significantly down-regulated, resulting in a reduction of AflS/AflR ratio. The results suggest that *A. niger* could directly inhibit AFB_1 biosynthesis through reducing the abundance of *aflS* to *aflR* mRNAs. Interestingly, atoxigenic *A. flavus* JZ2 and GZ15 effectively degrade AFB_1. Two new metabolites were identified and the key toxic lactone and furofuran rings both were destroyed and hydrogenated, meaning that lactonase and reductase might be involved in the degradation process.

1. Introduction

Aspergillus flavus is one of the most frequently isolated mold species in agriculture and medicine and a saprophytic filamentous fungus that is distributed all over the world especially in warm and moist fields (Cleveland et al., 2009). As a contaminant of stored grains, other crops and feeds, *A. flavus* produces an abundance of diverse secondary metabolites, such as aflatoxins and cyclopiazonic acid (CPA). Of them, the best known group of metabolites is aflatoxin, the most potent naturally occurring toxic and hepatocarcinogenic compounds (Squire, 1981). Aflatoxin is estimated to cause up to 28% of the total worldwide cases of hepatocellular carcinoma (HCC), the most common form of liver cancer (Wu, 2014). In addition to liver cancer, consumption of aflatoxin-contaminated foods and feeds can cause acute poisoning, immune-system dysfunction and stunted growth in children. People whose livers are already compromised by infection with hepatitis B virus (HBV) are particularly susceptible to aflatoxin-induced liver cancer (Groopman et al., 2008). Studies by Wu and her team (Wu, 2014) suggest that up to 172,000 cases of HCC per year can be attributed to exposure to aflatoxin in the diet, and most of these individuals are infected with HBV. The majority of cases occur in sub-Saharan Africa, Southeast Asia and the Western Pacific region (including China), as well as in parts of Central America (Wu, 2014). *A. flavus* also can cause direct infection and systematic disease in humans. After *Aspergillus fumigatus*, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis in immunocompromised patients (Cleveland et al., 2009). So, *A. flavus* and its metabolite aflatoxin not only are one of the important threats to human health, but also cause significant economic losses in many countries.

To prevent, control and eliminate harmful aflatoxin in foods for human consumption, numerous strategies have been utilized to control fungal growth and aflatoxin production (Amaike and Keller, 2011), and remove or degrade aflatoxin in products (Shcherbakova et al., 2015). These include the prevention of fungal infection on crops by applying atoxigenic biocompetitive *A. flavus* and/or *Aspergillus parasiticus* strains or yeast (Chang et al., 2012), enhancing host resistance, postharvest control of fungal growth, and prevention of aflatoxin production by using microorganisms and natural products (Ding et al., 2015; Liang et al., 2015). Due to efficiently eliminating toxins and safe-guarding the quality of food and feed, biological control of aflatoxin provides an attractive alternative. Except for atoxigenic *A. flavus* and *A. parasiticus*, many other filamentous fungi such as *Aspergillus chevalieri*, *Aspergillus*

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candidus, *Aspergillus oryzae* and *Aspergillus niger* could inhibit aflatoxin accumulation (Cvetnic and Pepeljnjak, 2007). Of these filamentous fungi, non-toxigenic *A. niger* isolated from food has the best prospective due to its safety (Xu et al., 2013). Nevertheless, to date, little is known about the mechanism of inhibition of aflatoxin B_1 (AFB_1) production and degradation of AFB_1 by *A. niger*.

The genes encoding the pathway for aflatoxin biosynthesis are within a 75 kb cluster in *A. flavus*. Up to now, 34 genes have been identified as members of the aflatoxin pathway gene cluster (Yu et al., 2004). Most of their functions in the pathway have been elucidated (Cleveland et al., 2009). Certain microorganisms and natural compounds can inhibit aflatoxin production by reducing the expression of the aflatoxin gene pathway. For example, co-cultivation of *A. flavus* with *Bacillus megaterium* down-regulates the expression of *aflF*, *aflT*, *aflJ*, *aflL* and *aflX* (Kong et al., 2014); 2-phenylethanol from *Pichia anomala* down-regulates the structural genes (*aflC*, *aflD*, *aflO* and *alfM*) involved in aflatoxin biosynthesis (Hua et al., 2014); curcumin inhibits AFB_1 production in *A. parasiticus* by down-regulating the aflatoxin pathway genes (*aflM*, *aflD*, *aflC*, *aflP* and *aflR*) (Jahanshiri et al., 2012); *Zataria multiflora* Boiss. essential oil down-regulates the gene expression of *aflD*, *aflM* and *aflP* in *A. parasiticus* (Yahyaraeyat et al., 2013); and cinnamaldehyde, citral and eugenol down-regulate the expression of *aflR*, *aflT*, *aflD*, *aflM* and *aflP* (Liang et al., 2015).

The aim of the present work was to investigate the mechanism behind the inhibitory role of *A. niger* on aflatoxin production by *A. flavus*, molecular expression profile of two key regulatory genes *aflR* and *aflS*, and 18 biosynthetic structural genes in pathway cluster, and the key regulatory gene of secondary metabolite *laeA*, and transcriptional activator genes of asexual sporulation *brlA*, were analyzed using real-time PCR. In addition, the effect of *A. niger* filtrates on aflatoxin production was also characterized by using the Spin-X centrifuge system. The AFB_1 degradation activity of atoxigenic GZ15 and JZ2 was investigated. And the degradation products and pathway were characterized.

2. Materials and methods

2.1. Fungal strains and culture conditions

The aflatoxigenic strain of *A. flavus* YC15 (high AFB_1 producer) was used as the pathogenic fungus. *A. flavus* JZ2 and GZ15 were isolated from peanut fields and proved in our lab to be atoxigenic strains without some key aflatoxin biosynthetic genes. Twenty strains of *A. niger* were isolated from peanut kernels in our lab (Ding et al., 2015). These strains were maintained on potato dextrose agar (PDA) medium (containing the extract of 200 g boiled potato, 20 g glucose and 20 g agar in 1 L of distilled water) at 4 °C. Conidial suspensions were harvested from sporulated cultures (7-day-old) of fungi on PDA plates by surface washing with a 0.01% Tween-20 solution in sterile deionized water. Conidia were counted with a hemocytometer and adjusted to 1×10^6 conidia/mL with 0.01% Tween-20 solution. For studying the aflatoxin biosynthetic gene expression level, fungal conidia were inoculated into 150 mL flasks containing 50 mL Yeast Extract Sucrose broth (YES, 20 g yeast extract, 150 g sucrose, and 0.5 g $MgSO_4 \cdot 7H_2O$ in 1 L of deionized water) and grown at 28 °C on a rotary incubator at 150 rpm for 5 d.

2.2. Preparation of the culture filtrate of A. niger

The culture filtrate of *A. niger* was prepared according to the method described by Xu et al. (2013) with minor modifications. Ten milliliters of a conidial suspension from a strain of *A. niger* was added to 500 mL of potato dextrose broth (PDB) and incubated at 28 ± 2 °C for 5 days with shaking at 150 rpm. The mycelia were separated from the substrate by filtration with four layers of cheese cloth and the culture filtrates were concentrated 10-fold by ultrafiltration (membrane cut off 1 kDa). Then the concentrated culture filtrate was sterilized using 0.2 μm disposable syringe filters (Millipore, Bedford, MA, USA) for the following experiments.

2.3. Effect of A. niger on the growth of A. flavus and aflatoxin production

2.3.1. Effect of co-cultivation of A. niger on the growth of A. flavus and AFB_1 production

The conidial suspensions of *A. niger* were adjusted to 1×10^6 conidia/mL with sterile 0.1% tween-20 solution, respectively. YES broth (50 mL) containing 1 mL of 1×10^6 conidia/mL suspensions of *A. flavus* was respectively inoculated with 1 mL of suspensions of *A. niger* conidia at different concentration and incubated in the dark at 28 ± 2 °C for 15 days with shaking at 150 rpm. Control without the conidia of *A. niger* was carried out under the same conditions. All treatments were tested in triplicate. The fungal growth and AFB_1 production in the culture medium were assayed every three days of incubation.

2.3.2. Effect of the culture filtrate of A. niger on radial growth of A. flavus

Influence of the culture filtrate of *A. niger* on radial growth of *A. flavus* mycelium was assayed according to the method described by Gandomi et al. (2009) with minor modification. The concentrated culture filtrate was added to molten PDA medium with the concentration of 2% (v/v). A 5 mm sterile diameter Whatman No. 1 filter paper disc was placed at the center of each plate and inoculated with 10 μL of *A. flavus* conidia suspension. Plates were incubated for 7 days at 28 ± 2 °C in the darkness. For the control, only PDA medium was used. All treatments were tested in triplicate. The diameter of colony was measured in two directions at right angles to each other to obtain the mean diameter.

2.3.3. Effect of culture filtrate on growth of A. flavus and AFB_1 production

YES (50 mL) containing 2% (v/v) of the concentrated culture filtrate was inoculated with 1 mL of conidia suspensions of *A. flavus* and incubated in the dark at 28 ± 2 °C on a rotary shaker (150 rpm) for 15 days. YES without the culture filtrate was incubated under the same conditions as the control. The fungal growth and AFB_1 in the culture medium were assayed every three days of incubation.

2.3.4. Effect of culture filtrate of A. niger on AFB_1 production by A. flavus using Spin-X centrifuge filter system

Conidial suspensions were diluted to 1×10^6 conidia/mL mixed with YES medium. The effect of *A. niger* on the aflatoxin production was assayed according to the method described by Huang et al. (2011) with minor modifications. Conidial-medium suspensions (500 μL of 1×10^6 conidia/mL) were placed in the filter tube insert above a 0.45 μm cellulose acetate filter in the Spin-X tube. As shown in Fig. 2, four treatments with four replications were included: 1) *A. niger* to exchange, 2) *A. flavus* to exchange, 3) *A. niger* and *A. flavus* together, 4) *A. flavus* alone. The tubes were incubated in an Eppendorf microfuge at 28 °C and centrifuged at 5000 rpm (2000 g) for 1 min every 3 h for 6 days. At the end of each centrifugation the insert, now free of liquid but containing growing fungus, was removed from the tube. In the last two treatments the medium contained in the tube was poured back into the insert from which it came, placed back in the tube, capped and mixed on a vortex before placing back in the centrifuge to incubate another 3 h. The first two treatments were done in the same way except the filtrate in the tubes from *A. niger* isolate was placed into the inserts containing *A. flavus* and vice versa. Every 3 h the first two treatments were exchanged so the *A. flavus* isolate grew for 3 days of the whole duration of the experiment in broth medium in which *A. niger* isolate had grown. Then, they were again centrifuged, and 600 μL methanol was added, the tube capped and vortexed before pouring over an alumina column into a vial for HPLC analysis of AFB_1. All of them were filtered using 0.22 μm disposable syringe filters for HPLC detection.