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Effect of water activity and temperature on the growth of *Aspergillus flavus*, the expression of aflatoxin biosynthetic genes and aflatoxin production in shelled peanuts

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ABSTRACT

The contamination of peanuts with *Aspergillus flavus* and subsequent aflatoxins is considered to be one of the most serious safety problems in the world. Water activity (a_w) and temperature are limiting factors for fungal growth and aflatoxins production during storage. To optimize the practical storage parameter, the effect of a_w (0.85–0.99) and temperature (15–42 °C) on fungal growth, aflatoxin production and the expression of aflatoxin biosynthetic and regulatory genes in shelled peanuts was investigated. *A. flavus* grew at a lower rate when temperature ≤20 °C or $a_w \leq 0.85$. For the growth of *A. flavus* in shelled peanuts, the optimum conditions were a_w was 0.98, and the optimum temperature was 37 °C. The maximum amount of AFB_1 in peanuts was obtained at 28 °C and a_w 0.96. Real-time analysis showed that 16 of 25 genes had highest expression levels at 28 °C under a_w 0.92, while 9 genes had highest expression levels at 37 °C under a_w 0.92. Compared with 37 °C, all aflatoxin biosynthetic pathway genes were down-regulated at 42 °C. All the pathway genes and *laeA* were up-expressed at a_w of 0.96 under 28 °C, compared to a_w 0.99. Furthermore, there was a good positive correlation between the ratio of *aflS/aflR* and AFB_1 production. The expression of *laeA* was also positively correlated with AFB_1 production while the expression of *brlA* was correlated with the *A. flavus* growth. The results of this study suggest that AFB_1 production in peanut kernels can occur over a wider range of $a_w \times$ temperatures levels compared to formula media and peanut media. Previous studies have showed that AFB_1 could not be produced on formula media at 37 °C without the expression of most aflatoxin structural genes. But, in the un-autoclaved shelled peanuts, high concentration of AFB_1 was produced at 37 °C with up-regulation of some aflatoxin biosynthetic genes. From a food safety point of view, the results can be used to optimize certain food technological processes and develop prevention strategies to control such carcinogenic natural metabolites in grains (such as peanuts, maize and rice) and derived products.

1. Introduction

Peanut (*Arachis hypogaea* L.) is a globally important economic and oilseed crop in the world, which is cultivated and harvested at large scale, with Africa continent, China and India being the greatest producers. However, peanuts contamination with aflatoxins and aflatoxigenic *Aspergillus* is considered as the most serious problem in the world (Bankole & Adebanjo, 2004; Williams et al., 2004). Aflatoxin contamination compromises the quality of the product as they are highly toxic and carcinogenic compounds among the toxins. Aflatoxin B_1 (AFB_1), which poses a health risk in animals and humans, has been classified as a class I human carcinogen by the International Agency for Research on Cancer (IARC, 1993, 2002). Aflatoxin is estimated to cause up to 28% of the total worldwide causes of hepatocellular carcinoma (HCC), the most common form of liver cancer (Liu & Wu, 2010; Liu, Chang, Marsh, & Wu, 2012; Wu, 2014). People who have liver infection with hepatitis B virus (HBV) are particularly susceptible to aflatoxin-induced liver cancer (Groopman, Kensler, & Wild, 2008).

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Due to its high threat to the health of humans and animals, more than 100 countries and organizations including the National Health and Family Planning Commission P.R. of China, the European Union and the U.S. Food and Drug Administration, have established limits for total aflatoxins and AFB_1 levels in peanuts (Commission of the European Communities, 2010; US Food and Drug Administration, 2010).

Aflatoxins are mainly produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins biosynthesis is a complex enzymatic reaction that has been extensively studied (Cleveland et al., 2009; Yu et al., 2008). As shown in Fig. 1, the genes encoding the aflatoxin biosynthesis pathway are within the 75 Kb gene cluster in *A. flavus*. To date, 29 genes have been identified as members of the aflatoxin pathway gene cluster and their functions have been elucidated. *A. flavus* and *A. parasiticus* are exposed to several environment conditions, and aflatoxin production is markedly affected by several external factors and growth conditions, such as temperature, water activity, pH and carbon and nitrogen source (Schmidt-Heydt, Abdel-Hadi, Magan, & Geisen, 2009). Especially, water activity (a_w) and temperature are limiting factors in the functioning of the storage ecosystems (Giorni, Magan, & Battilani, 2009). It has been shown that both a_w and temperature modifications affect the relative growth rate, aflatoxin production and the expression of aflatoxin pathway genes in both *A. flavus* and *A. parasiticus* (Schmidt-Heydt, Rüfer, Abdel-Hadi, Magan, & Geisen, 2010; Schmidt-Heydt, Parra, Geisen, & Magan, 2011). It was shown that temperature $\times$ a_w interactions were related to the ratio of the two key regulatory genes (*aflS*/*aflR*). The higher ratio between *aflS* and *aflR* would relate to higher AFB_1 production (Schmidt-Heydt, Abdel-Hadi, Magan, & Geisen, 2009; Schmidt-Heydt et al., 2010). Similarly, Yu et al. (2011) examined the effect of elevated temperature on the relative expression of the whole genome of a type strain of *A. flavus* to identify groups of up and down regulated genes. They found that high temperature negatively affects aflatoxin production by turning down transcription of the two key transcriptional regulators, *aflR* and *aflS*. Subtle changes in the expression levels of *aflS*/*aflR* appear to control transcription activation of the aflatoxin cluster. These results suggest that under certain interacting conditions of two environmental stress factors could significantly affect the relative AFB_1 production.

However, most of the above results were obtained by incubating *A. flavus* on formula media or peanuts media. The effect of a_w and temperature on *A. flavus* growth and aflatoxin production on formula media is very different from that in peanuts and grains. To determine the practical storage parameters, the effect of a_w and temperature on the growth of *A. flavus,* aflatoxin production and the expression of aflatoxin biosynthetic pathway genes in unautoclaved shelled peanuts was investigated. For the expression of aflatoxin biosynthetic genes, the key two regulatory genes *aflR* and *aflS*, and 20 biosynthetic structural genes in pathway cluster, and the key regulatory gene of secondary metabolite *laeA*, and transcriptional activator gene of asexual sporulation *brlA*, and the differentiation gene of phialides *abaA*, were analyzed using real-time PCR. The aflatoxin biosynthetic pathway genes used for real-time PCR analysis are shown in Fig. 1 and marked with blue underline.

2. Materials and methods

2.1. Chemicals

High performance liquid chromatography (HPLC) grade methanol and acetonitrile were purchased from Fisher Scientific (Fisher Chemicals HPLC, USA). AFB_1 standard was procured from Sigma-Aldrich Chemicals (USA). ToxinFast immunoaffinity columns for AFB_1 were purchased from Huaan Magnech Bio-tech (Beijing, China).

2.2. Fungal strain and growth conditions

The aflatoxigenic strain of *A. flavus* YC15 (high AFB_1 producer) was used as the pathogenic fungus. The strain was 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. Spore suspensions were harvested by surface washing of sporulated cultures (7-day-old) of fungi in malt extract agar (MEA) medium (containing 30 g malt extract, 3 g soy peptone and 20 g agar in 1 L of distilled water) with a 0.01% Tween-20 solution in sterile deionized water. Spores were counted with a hemocytometer and adjusted to 1×10^6 conidia/mL with 0.01% Tween-20 solution.

2.3. Inoculation of peanut samples

The inoculation of peanut samples with *A. flavus* was conducted according to the method described by Abdel-Hadi, Schmidt-Heydt, Parra, Geisen, and Magan (2012) with minor modifications. A moisture adsorption curve was prepared for shelled peanuts to accurately determine the amount of water required to add to obtain the targeted a_w levels. This curve was obtained by adding different quantities of water to peanuts, equilibration overnight, and then determining moisture content by 130 °C for 12 h, and comparing this with the a_w level measured with an Aqualab 4TE (Decagon Devices, Pullman, WA, USA).

Shelled peanuts were disinfected for 3 min by 0.1% sodium hypochlorite, followed by rinsing thrice with sterile distilled water. And then required water was added to obtain the targeted a_w levels (0.85, 0.90, 0.92, 0.94, 0.96, 0.98 and 0.99). The ranges of a_w and temperature were established according to the study by Abdel-Hadi et al. (2012) with some modifications. After equilibration, one hundred grams of disinfected peanuts was put in autoclaved flasks covered with lids containing a microporous membrane. The peanut samples were inoculated with 1 mL of 1×10^6 conidia/mL suspensions of *A. flavus* and vigorously shaken to coat the peanuts with spores. Then, the peanut samples were incubated at different temperature (15, 20, 25, 28, 33, 37, 40 and 42 °C) for 7 days in polyethylene sandwich boxes containing glycerol/water solutions to maintain the equilibrium relative humidity conditions in a constant temperature and humidity incubator. For negative controls, the peanut samples without inoculating with *A. flavus* were incubated at the same conditions. Each flask was shaken once a day. Three experimental replicates were performed for each treatment, and three biological replicates were performed for every experiment.

2.4. Determination of colony-forming units (CFUs)

The *A. flavus* total CFUs were determined by serial dilution and spread plating the different dilutions on MEA plate and incubating for 4–5 days before counting the numbers of colonies.

2.5. Determination of aflatoxin B_1

A 50 g sub-sample of peanut kernels after sterilization at 121 °C for 30 min was finely ground with a grinder, and stored at 4 °C in suitable glass container before determination of AFB_1. AFB_1 in the finely ground samples was extracted and detected by the HPLC according to the method by Chinese standard methods and the AOAC method 994.08 (AOAC, 2000) with minor modifications. Firstly, 25 g ground samples were extracted with 125 mL of