Rapid isolation of non-aflatoxigenic *Aspergillus flavus* strains

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Abstract

In the present study, a method for screening non-aflatoxigenic *Aspergillus flavus* in soil samples collected from major peanut-growing regions of China was developed. The single colonies were picked and cultured on *Aspergillus flavus* and *parasiticus* agar (AFPA). If the reverse side of the colony on AFPA was orange-coloured, it was considered *A. flavus* or *Aspergillus parasiticus*. After the genomic DNA of each strain was extracted, 28S rRNA and calmodulin were amplified and sequenced to determine the species. The key gene, *aflR*, was amplified and digested via polymerase chain reaction-restriction fragment length polymorphism. The aflatoxigenic *A. flavus* and the non-aflatoxigenic *A. flavus* and *A. parasiticus* were distinguished by enzyme digestion of *aflR*. 156 strains of *A. flavus* were screened, which consisted of 135 aflatoxigenic and 21 non-aflatoxigenic strains. The aflatoxin producing ability of each strain was confirmed using solid-state fermentation experiments. Using the method developed in the present study, we confirmed that the non-aflatoxigenic *A. flavus* strains isolated lost their capacity to produce aflatoxins. Considering there could be some alterations in other functional genes, some non-aflatoxigenic strains could be identified inaccurately as aflatoxigenic strains, although that did not occur in the present study. The growth of non-aflatoxigenic *A. flavus* was observed, and the most rapidly growing non-aflatoxigenic strain was selected for plate confrontation assays and toxic mixed culture experiments. The inhibition rate of non-aflatoxigenic *A. flavus* against aflatoxigenic *A. flavus* was 55.4 and 72.6% in potato dextrose agar (PDA) plate and natural soybean medium, respectively. The screened non-aflatoxigenic *A. flavus* strains provide a microbial resource for biological control of aflatoxin contamination.

Keywords: aflatoxins, *aflR*, PCR-RFLP, solid-state fermentation, inhibition

1. Introduction

Aflatoxins (AFs) are a group of secondary metabolites in *Aspergillus* fungi, such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Kowalska *et al.*, 2017; Varga *et al.*, 2009). AFs have been listed as the most dangerous mycotoxins in food due to their high carcinogenic and teratogenic toxicity. For instance, the carcinogenicity of AFB₁ is 75-fold of that of the standard carcinogen dimethyl nitrosamine (Horn and Dorner, 1998; IARC, 1993) and 10- and 68-fold of that of potassium cyanide and arsenic, respectively. The intake of only 0.294 mg/kg AFB₁ would subject sensitive animals to acute poisoning and death (Li *et al.*, 2009). In 1994, the International Agency for Research

on Cancer (IARC) listed AFB₁ as a Class I carcinogen (Horn and Dorner, 1999). Consequently, the European Union issued a unified food aflatoxin limit standard in its member states and set the limits of AFB₁ and total AF (aflatoxin B₁ + B₂ + G₁ + G₂) at 2 and 4 µg/kg, respectively, in peanuts and other foods (EC, 2006).

AFs exhibit remarkable stability and hardly degrade under food-processing environments (Liu and Wu, 2010). The occurrence and accumulation of AFs in peanut and other cereals have caused major economic losses globally. Consequently, strategies for reducing aflatoxin pollution in the course of crop development are urgently required.

Introducing non-toxicogenic *A. flavus* or *A. parasiticus* to microbial flora in soils opens a new horizon for controlling AF-producing microorganisms, which has been reported to be remarkably effective in reducing AF-contamination prior to harvest (Bandyopadhyay *et al.*, 2016; Dorner and Lamb, 2006). As early as the 1990s, a study proposed the introduction of non-toxicogenic strains of *A. flavus* in soil in order to reduce the aflatoxin concentrations in cotton seeds (Cotty, 1990). In addition, Dorner *et al.* (1992) pointed out that the spread of non-toxicogenic *A. parasiticus* strains in peanut-growing soil could decrease the aflatoxin concentrations in peanuts by 83-85% (Dorner *et al.*, 1992). At present, commercialised non-toxicogenic strains of *A. flavus* are registered in numerous countries. For example, the US Environmental Protection Association has registered two non-toxicogenic strains of *A. flavus* to control cotton and peanut aflatoxin contamination, and both have been extensively tested in experimental fields in several states in the United States (Cardwell *et al.*, 2008). In Africa, Aflasafe™ has been produced using a mixture of four non-toxicogenic strains (Bandyopadhyay *et al.*, 2016). In Italy, the commercial biocontrol product, AF-X1, which originates from the non-toxicogenic *A. flavus* strain MUCL54911, is currently used in maize (Mauro *et al.*, 2018). In Australia and China, strains of non-toxicogenic *A. flavus* that can effectively inhibit aflatoxin production have also been screened, and the strains have reduced the number of toxin-producing strains by more than 95% (Pitt and Hocking, 2006; Yin *et al.*, 2008). Introducing non-toxicogenic *A. flavus* and *A. parasiticus* to control the microbial flora in the soil could lead to the replacement of the original toxin-producing strain in the soil in the course of crop growth, which is very effective in reducing aflatoxin contamination prior to harvesting (Dorner and Lamb, 2006). Dorner and Horn (2007) observed that *A. flavus*, as opposed to *A. parasiticus*, is more likely to colonise peanuts in soil (Dorner and Horn, 2007).

The key aspect of the strategy is the rapid screening and identification of competitive non-aflatoxigenic *A. flavus* strains. The identification of *Aspergillus* section *Flavi* strains has been based on polyphasic approaches, including chemical, morphological and molecular characterisation (Paula *et al.*, 2009).

Barros *et al.* (2010) used AFLP to compare 82 isolates of *Aspergillus* section *Flavi* and clearly separated *A. flavus* and *A. parasiticus* using cluster analysis. The comparison of fingerprints also revealed several specific markers for each group of isolates; however, no genotypical differences in passage were observed between aflatoxigenic and non-aflatoxigenic producers (Barros *et al.*, 2010).

Aflatoxin synthesis involves a complex 21-step enzymatic reaction process (Bhatnagar *et al.*, 2003). Most of the genes involved in the synthesis are in specific gene clusters, which are transcribed and translated by genes of the

gene clusters (Yu *et al.*, 2000). Gene clusters are common secondary metabolisms in cells (Keller and Hohn, 1997). The presence of gene clusters is one of the key mechanism of regulation of the aflatoxin biosynthesis pathway and other secondary metabolic pathways (Georgianna and Payne, 2009). *AflR*, a key gene in the aflatoxin biosynthesis pathway, plays an important role in toxin production. In addition, as a key protein for aflatoxin synthesis and a major positive transcription factor, the AflR protein regulates the transcription of other genes in the aflatoxin biosynthesis pathway (Ehrlich *et al.*, 1998, 2003; Georgianna and Payne, 2009; Rokas *et al.*, 2007; Yu *et al.*, 2002). Most aflatoxin synthesis related genes are regulated by the AflR protein (Bhatnagar *et al.*, 2006; Galagan *et al.*, 2005; Matsushima *et al.*, 2001; Nierman *et al.*, 2005; Price *et al.*, 2010). Some mutation points in the *aflR* promoter region were revealed in none-AFB₁-producing *A. flavus* strains via gene analysis using OMIGA (Oxford Molecular Ltd., Cambridge, UK), and one or some of the mutation sites could be associated with aflatoxin production (Chen and Liu, 2006).

The aim of the present study was to develop a novel and efficient method for rapid isolating non-aflatoxigenic strains of *A. flavus* and to offer a microbial resource for the biological control of aflatoxin contamination.

2. Materials and methods

Fungal isolates and culture conditions

Soil samples were collected from four major peanut-growing regions of China, including Tangshan City in Hebei Province, Zhengyang and Wuyang City in Henan Province, Xiangyang City in Hubei Province, in 2017 and 2018. Under sterilised conditions, 10 g of each sample was placed in a 250 ml conical flask and 90 ml of 0.1% sterile peptone water solution (w/v) added. The mixture was sealed and shaken for 30 min. Subsequently, 1 ml of the solution was mixed in a centrifugal tube containing 9 ml of 0.1% peptone sterile water, forming a 1:100 dilution solution (Zanon *et al.*, 2013). Based on the procedure, a gradient sample dilution solution was prepared using 10-fold diluted gradient dilution; 100 µl of 10⁻² and 10⁻³ gradient soil dilution solution was added to Dicloran 18% glycerol medium salt Czapek Dox (CZ) agar (DG18). The plate was placed upside down in an incubator and cultured at 30 °C for four days. Subsequently, a single colony was picked and transferred to a new DG18 plate. After three days of culture, the spores were transferred into 20% glycerol and stored at -80 °C for subsequent analyses.

Twelve *Aspergillus* strains, including two *A. parasiticus* strains, eight *A. flavus* strains, one *Aspergillus oryzae* strain and one *Aspergillus sojae* strain, were used as the internal references. Their identities, numbers and origins are listed in Supplementary Table S1. All isolates were maintained in 20% glycerol at -80 °C.

Phenotypic characterisation

Each isolate was cultivated on *Aspergillus flavus* and *parasiticus* agar (AFPA) purchased from Qingdao Haibo Biotechnology Co., Ltd. (Qingdao, China P.R.; peptone 10 g/l; yeast soaking powder 20 g/l; ferric ammonium citrate 0.5 g/l; Dichloran 0.002 g/l; chloramphenicol 0.1g/l; agar 15 g/l; pH 6.3±0.2) and cultured at 30 °C for 72 h in the dark. Both *A. flavus* and *A. parasiticus* produce aspergillilic acid, which reacts with ferric citrate in AFPA medium to generate an orange pigment (Baquiao *et al.*, 2013). Each strain was inoculated on a petri dish containing Czapek agar and incubated at 30 °C for 14 days. If no sclerotia were observed under the conditions, the strain was not considered sclerotia-producing. Ten sclerotia were randomly selected in each medium for the observation of diameter, shape and colour. Sclerotia with diameter <400 µm and >400 µm were classified as S-type and L-type, respectively (Cotty, 1989).

DNA extraction and amplification

DNA was extracted using the GV-filamentous fungi genomic DNA extraction kit (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China P.R.) according to the manufacturer's instructions. Two regions of the genome were named the 28S *rRNA* and the partial calmodulin gene. The 28S *rRNA* was amplified using the *NL₁/NL₄* primer pair that was designed based on the sequences of aflatoxin biosynthesis genes in *A. flavus* (GenBank accession No. AY510451.1) (Jiang *et al.*, 2009). The calmodulin gene was amplified using the *CL₁/CL_{2A}* primers (O'Donnell *et al.*, 2000). The amplification products were sequenced by a biotech company (BGI, Shenzhen, China P.R.), and then the sequences were aligned using data from the US National Center for Biotechnology Information database, which classified the species preliminarily. The sequences of the primers are listed in Table 1.

Table 1. Sequences of primers and probes used in the study.

Name	Sequences
<i>aflR₁</i>	5'-AGAATAGCTTCGACGGGTGGT-3'
<i>aflR₂</i>	5'-AGTCTGGGAGGAACGGATCG-3'
<i>PaflR₁</i>	5'-AACCGCATCCACAATCTCAT-3'
<i>PaflR₂</i>	5'-AGTGCAGTTCGCTCAGAACA-3'
<i>NL₁</i>	5'-TGCGTTGATTACGTCCTGC-3'
<i>NL₄</i>	5'-GGTCCGTGTTTCAAGACGG-3'
<i>CL₁</i>	5'-GARTWCAAGGAGGCCCTTCTC-3'
<i>CL_{2A}</i>	5'-TTTTTGCATCATGAGTTGGAC-3'

PCR-RFLP of the *aflR* gene

The primer pairs were designed based on comparisons of *aflR* and its promoter involved in the aflatoxin biosynthesis among *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae*. The *aflR* and promoter gene were amplified using the *aflR₁/aflR₂* and *PaflR₁/PaflR₂* primer pairs, respectively (Table 1). The PCR-RFLP results of the twelve *Aspergillus* fungi listed in Supplementary Table S1 are presented as examples.

PCR was conducted using a C1000 PCR instrument (BioRad, Hercules, CA, USA). The 25 µl PCR system consisted of 12.5 µl of Premix PrimeSTAR HS enzymes, 0.5 µl of upstream primers, 0.5 µl of downstream primers and 10.5 µl of ultra-pure water. To obtain pure PCR products, we designed a special PCR process to apply the primer annealing temperature cumulatively. The reaction system was processed as follows: preheating at 98 °C for 5 min; 30 cycles of 30 min at 98 °C, 30 s at 52 °C, 1 min at 72 °C and extension at 72 °C for 10 min. The PCR products were detected using 1.5% gel electrophoresis.

Restriction sites were analysed using SnapGene (GSL Biotech, Chicago, IL, USA). The enzyme *NheI* cleavage system was performed in a 20 µl volume containing 5 µl of PCR product, 2 µl of 10× FastDigest Green Buffer, 1 µl of FastDigest enzyme and 12 µl of Milli-Q water. The system was also suitable for the other two enzymes including *HincII* and *PvuII*. All reaction systems were incubated at 37 °C for 10 min in a digital dry bath and then inactivated by heating to 65 °C for 5 min. The products were assessed on 1.5% agarose gels in Tris-acetate buffer and photographed after staining with a nucleic acid dye, GelStain (TransGen Biotech, Beijing, China P.R.). The strains were classified based on the results of three cleavage reactions.

High-performance liquid chromatography for aflatoxins

In total, 156 *A. flavus* isolates and two *A. parasiticus* strains were selected for the evaluation of aflatoxin production ability when cultured using natural soybean substrates. AFB₁, AFB₂, AFG₁ and AFG₂ aflatoxin production abilities were determined using HPLC according to Shotwell *et al.* (1966) with slight modification. Each strain was inoculated on 75 g autoclaved soybean and 25 ml sterile water in a 250 ml Erlenmeyer flask with a 1.5 ml conidial suspension rate that was obtained from the DG18 medium after 7-day culture. The cultures were incubated at 30 °C in the dark for 27 days.

At the end of the incubation, the soybean was autoclaved at 121 °C for 30 min to inactivate the strains. The cultures were ground using a high-speed crusher, and then 5 g of the mixture was added into a 125 ml Erlenmeyer flask with 1 g NaCl. Afterwards, 100 ml of methanol-water (70:30, v/v) was added to extract the aflatoxins. After

high-speed homogenisation, the liquid-phase extract was filtered through a layer of rapid qualitative philtre paper. Subsequently, 10 ml of the filtrate was mixed with 20 ml of water and the mixture was filtered using microfiber philtre paper, and then 15 ml of the filtrate was added into an aflatoxin B₁/B₂/G₁/G₂ immunoaffinity column (Beijing Huaan Maiké Biotechnology Co., Ltd., Beijing, China P.R.).

When the toxin content in the sample was higher than the column capacity (300 ng), the sample volume was reduced appropriately, or the dilution factor was increased. Finally, the AFs were dissolved in 1 ml methanol.

Afterwards, the mixture of the dissolved AFs was injected into an Alliance e2695 HPLC system (Waters, Milford, MA, USA) equipped with a 2475 fluorescence detector (excitation 365 nm, emission 450 nm) and an autosampler system. The loading volume was 10 µl. An improved photochemical reactor (AURA, New York, NY, USA, 230 Volt, 50 Hz, 8 Watt) was installed between the analytical column (Waters, XBridge C18 5 µm, 4.6×250 mm) and the fluorescence detector. The isocratic mobile phase contained methanol: water (1:1, v/v) and was eluted at a flow rate of 0.5 ml/min.

Data were normalised according to the calibration curves of standard solutions of AFB₁ and AFG₁ (both 1000, 500, 300, 100, 10 and 2.0 ng/ml) and AFG₂ and AFB₂ (both 300, 150, 90, 30, 3 and 0.6 ng/ml). The detection and quantification limits were 0.5 and 0.75 ng/ml, for AFB₁ and AFG₁, respectively, and were 0.15 and 0.225 ng/ml, respectively, for AFB₂ and AFG₂.

The mycelium growth rate and confrontation assay

The non-aflatoxigenic *A. flavus* isolated in the study and aflatoxigenic *A. flavus* that produced the highest AFB₁ amounts were cultured on potato dextrose agar (PDA) plates and incubated at 25 °C for 9 days. Colony diameters of each plate were observed. Three replicates were included for each strain.

The non-aflatoxigenic *A. flavus* strains that grew most rapidly based on colony diameter after incubation for 72 h were cultured on PDA agar and natural soybean substrates together with the aflatoxigenic *A. flavus* that produced the highest AFB₁ quantities. Non-toxicogenic *A. flavus* and toxicogenic *A. flavus* were inoculated at the same time on the PDA plate (culture dish diameter: 8.5 cm) 2 cm from the centre on both sides. The aflatoxigenic *A. flavus* was inoculated on the same location of the PDA plate as a control group. Plate confrontation assay was performed at 30 °C and repeated thrice. The diameters of the strain colonies were observed daily. When the hyphae of the control culture filled the whole petri dish, the diameters of the tested non-aflatoxigenic *A. flavus* pointing to the

aflatoxigenic *A. flavus*, the diameters of the aflatoxigenic *A. flavus* pointing to the non-aflatoxigenic *A. flavus* and the diameters of the control aflatoxigenic *A. flavus* were measured respectively. The inhibition ratio was calculated according to Equation 1.

$$\text{Inhibition ratio (\%)} = \frac{\text{diameter of toxigenic } A. \text{ flavus in control} - \text{diameter of toxigenic } A. \text{ flavus in confrontation}}{\text{diameter of toxigenic } A. \text{ flavus in control}} \times 100 \quad (1)$$

The spore suspensions of toxigenic and non-toxicogenic *A. flavus* were prepared using 10⁶ cfu/ml concentrations. Using soybean as the culture medium, 1.5 ml aflatoxin producing and 1.5 ml non-aflatoxin producing spores were added as the experimental groups. In the control group, only the aflatoxin producing *A. flavus* spore solution was added. Three parallel experiments were performed for each group. AFB₁ presence in each group was evaluated after incubation at 30 °C for 27 days. The inhibition ratio was calculated according to Equation 2.

$$\text{Inhibition ratio (\%)} = \frac{\text{AFB}_1 \text{ production in control} - \text{AFB}_1 \text{ production in confrontation}}{\text{AFB}_1 \text{ production in control}} \times 100 \quad (2)$$

3. Results

Phenotypic characterisation

In total, 312 isolates of *Aspergillus* section *Flavi* were isolated from four major peanut-growing regions of China, and 156 strains were identified as *A. flavus* or *A. parasiticus* when the reverse side of the colony on AFPA was orange. In contrast, *A. oryzae* and *A. sojae* isolates exhibit yellow or brown colours on AFPA. Example plates of the four *Aspergillus* strains are presented in Supplementary Figure S1. The yyPAsp-nn designation code was employed, where yy, P, Asp and nn refer to the year, the commodity (peanut), the genus *Aspergillus* and the isolate special number, respectively.

Sequence analysis

Genetic identification of the isolates was achieved using genomic sequencing at two regions of the *Aspergillus* section *Flavi* genome. First, the results of all 28S *rRNA* and calmodulin sequences analysed using DNAMAN (Lynnon Biosoft, Quebec, Canada) suggested that similarity was over 95%. Secondly, based on the data in public databases searched using the BlastN algorithm, 28S *rRNA* and

calmodulin sequences showed more than 93% of identity with *A. flavus*.

PCR-RFLP results

PCR amplification and sequence analysis of *Aspergillus* strains demonstrated the amplified fragment was identical to the sequences of the designed *aflR* gene promoter (515 bp) and the *aflR* structural gene (798 bp) (Figure 1). The *aflR* promoter of the *Aspergillus* strains was tested using PCR and its sequences were analysed using DNAMAN. The sequences of the *aflR* promoter in *A. parasiticus* were similar, but had one more base when compared to *A. flavus*, *A. sojae* and *A. oryzae*. In addition, the promoter sequences among the non-toxicogenic *A. flavus*, *A. oryzae* and *A. sojae* were identical. *NheI* enzyme digestion analysis revealed that toxigenic *A. flavus* and *A. parasiticus* both had one restriction enzyme cutting site, while non-aflatoxigenic *A. flavus*, *A. oryzae* and *A. sojae* all had no *NheI* site (Supplementary Table S2). Analysis of *aflR* structural gene PCR amplification sequences revealed that *A. flavus* had two *HincII* enzyme cutting sites and *A. parasiticus* had one cleavage site, while *PvuII* of *aflR* structural gene showed that *A. flavus* had one restriction enzyme site and *A. parasiticus* had two restriction sites (Supplementary Table S3).

The *aflR* promoter fragment was digested with restriction endonuclease *NheI*; the *aflR* structural gene fragment was digested with restriction enzymes *HincII* and *PvuII* separately. The electrophoretic detection results are illustrated in Figure 1. The enzyme digestion results were

consistent with the bioinformatics analysis of GenBank resources and other studies (Somashekar *et al.*, 2004; Shao *et al.*, 2008).

Production of mycotoxins

Under the selected chromatographic conditions, the AFs were well separated. Supplementary Figure S2 shows the chromatograms of aflatoxin standards with 1000 ng/ml AFG₁ and AFB₁ and 300 ng/ml AFG₂ and AFB₂. Mycotoxins were produced by 86.5% (135/156) of the *Aspergillus* section *Flavi* isolates studied. Table 2 presents different aflatoxin quantification and aspergillic acid and sclerotium production trends of the *Aspergillus* section *Flavi* strains. Among the *A. flavus* screened (156), 135 (86.5%) of the strains produced AFB₁ and AFB₂ at 7.77 to 5,712.81 and 0.89 to 589.88 µg/kg, respectively. The production of AFB₁ was much higher than the production AFB₂. The average mycotoxin production levels are listed in Table 2. The two *A. parasiticus* strains both produced all the aflatoxins (AFG₂, AFG₁, AFB₂ and AFB₁), whereas *A. parasiticus* 3.0124 produced more aflatoxins than *A. parasiticus* JS (Table 2).

Sclerotium production

The presence, shape and size of sclerotia were observed after the strains were cultured in the CZ medium at 30 °C for 14 days. Sclerotia were produced by 125 of 156 *A. flavus* isolates, but by neither of the two *A. parasiticus* strains (Table 2). All the sclerotia were L-type (>400 µm), which were round to elongate. The average size of the sclerotia was 570±90 µm for *A. flavus* strains. About

Figure 1. Agarose gel electrophoresis of the target gene products and enzyme digestion results. (A) PCR amplification of the *aflR* promoter; (B) amplification of the *aflR* structural gene; (C) *aflR* promoters digested using *NheI*; (D) *aflR* digested using *HincII*; (E) *aflR* digested using *PvuII*. M = Marker DL1000; 1 = *Aspergillus parasiticus* 3.124; 2 = *A. parasiticus* JS; 3 = strain 18PAsp-xy1; 4 = strain 18PAsp-xy2; 5 = strain 18PAsp-ts2; 6 = strain 18PAsp-wy4; 7 = *Aspergillus flavus* 3.4408; 8 = non-aflatoxigenic *A. flavus* H1; 9 = non-aflatoxigenic *A. flavus* H2; 10 = strain 18PAsp-zy1; 11 = *Aspergillus oryzae* 3.484; 12 = *Aspergillus sojae* 3.880.

Table 2. Different aflatoxin quantification, aspergillic acid and sclerotium production trends of *Aspergillus* strains.

Species	Number of isolates (%)	Average mycotoxin production ¹ (µg/kg)					AA ²	Number of sclerotium producers (%)
		AFB ₁	AFB ₂	AFG ₁	AFG ₂	Total		
<i>A. flavus</i>	135 (86.5%)	936.72	87.69	ND	ND	1,024.41	+	108 (86.4)
<i>A. flavus</i>	21 (13.5%)	ND	ND	ND	ND	ND	+	17 (13.6%)
<i>A. parasiticus</i> 3.0124	– ³	430.02	108.47	1,630.88	214.27	2,383.64	+	0
<i>A. parasiticus</i> JS	– ³	130.13	46.54	321.72	74.38	572.77	+	0

¹ ND = not detected (<LOD).

² AA = aspergillic acid; (+) strains producing AA present an orange colour on the AFPA medium.

³ Only one strain.

108 of 135 aflatoxigenic *A. flavus* isolates and 17 of 21 non-aflatoxigenic isolates were sclerotium producers. In addition, *A. flavus* isolates producing sclerotia produced higher levels of aflatoxins (Supplementary Table S4).

Mycelium growth rate and confrontation assay

Water activity (a_w) of the PDA agar detected by a moisture activity tester (GYW-1GS; Guana, Shenzhen, China P.R.) was 0.952. The colony diameters increased linearly until the colonies reached the edge of the petri dishes. When the diameter was greater than or equal to 8.3 cm, the plates were considered covered with hyphae. Four strains of *A. flavus* filled the petri dishes on the sixth day. Furthermore, mycelia of almost all strains reached their maximum size on the eighth day. After cultivation for 72 h, the diameters of the non-toxicogenic strains varied from 3.7 to 4.6 cm. Fungal strain 18PAsp-zy1 was the fastest growing one. Figure 2 shows the growth curves of 18PAsp-zy1, 18PAsp-xy1 growing alone and 18PAsp-xy1 co-inoculated with non-toxicogenic *A. flavus*. When the colony diameter of 18PAsp-xy1 cultured alone in PDA agar was 8.3 cm, the diameter in the tested group was 3.7 cm. The distance between toxicogenic and non-toxicogenic strains of *A. flavus* was about 0.5 cm (Figure 3). The inhibition of the non-toxicogenic *A. flavus* on toxicogenic *A. flavus* was 55.4%. In the soybean culture, aflatoxin accumulation was reduced by more than 72% from 5,510.37 µg/kg to 1,509.84 µg/kg.

4. Discussion

It is challenging to distinguish *A. flavus* and *A. parasiticus* from *A. oryzae* and *A. sojae* in numerous media due to their similar phenotypic characteristics. Their spores are similarly yellow coloured in DG18 medium and yellow-green on CZ medium. We used the DG18 medium to select single isolates that resembled *A. flavus*. DG18 agar can retard or inhibit the growth of *Brettanomyces anomalus*,

Cryptococcus albidus and *Rhodotorula mucilaginosa*, which facilitates the selection of *Aspergillus* strains (Deak *et al.*, 2001). Subsequently, based on the unique orange colour on AFPA medium, we can easily distinguish *A. flavus* and *A. parasiticus* from other *Aspergillus Flavi* isolates.

A. flavus, *A. oryzae*, *A. parasiticus* and *A. sojae* belong to *Aspergillus* section *Flavi*. They all contain the *aflR* gene, and the *aflR* sequences are almost similar. We analysed the *aflR* promoter sequences and structural genes of *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* and observed that some of the bases changed regularly, resulting in changes of restriction sites, such as *NheI*, *PvuII* and *HincII*. The *aflR* promoter sequence of the aflatoxin producing strains harboured an *NheI* cleavage site that divided the *aflR* promoter into two fragments of 176 and 340 bp in length, while the cleavage site disappeared after the mutation, indicating that the toxin-producing isolates and the non-

Figure 2. Growth curves of *Aspergillus flavus* strains 18PAsp-zy1 (non-toxicogenic) and 18PAsp-xy1 (toxicogenic) growing alone and co-inoculated.

Figure 3. Toxigenic *Aspergillus flavus* strain 18PAsp-xy1 (left) co-inoculated with non-aflatoxigenic *A. flavus* strain 18PAsp-zy1 (right).

toxigenic isolates could be distinguished. The *aflR* gene was then digested with the *PvuII* enzyme and the *HincII* enzyme to distinguish *A. flavus* and *A. parasiticus*. Conventional PCR can separate *Aspergillus* using enzymatic digestion and agarose gel electrophoresis, and the results indicated that the gene sequences of toxigenic and non-toxicogenic fungi were different. In addition, the aflatoxin producing abilities were verified using solid-state fermentation experiments.

The objective of the present study was to screen the non-aflatoxigenic strains of *A. flavus* from soil samples. Toxin-producing ability is often assessed through solid-state fermentation, which requires almost a month, in addition to being easily affected by the environment. Using the PCR-RFLP method, we can evaluate the capacities based on gene levels. The method requires only approximately a week to obtain the non-aflatoxigenic isolates. Therefore, it is possible to ensure that the screened non-aflatoxigenic strains have lost the toxin-producing ability.

The PCR-RFLP method established in the present study only focuses on the *aflR* gene. There are numerous atoxigenic strains with a functional *aflR* gene. The patterns of deletions in the aflatoxin biosynthetic gene cluster appear to be common and diverse. Chang *et al.* (2005) found eight deletion patterns among which three patterns contained *aflR* gene. In 2016, genotypes were divided into 6 groups (Group A to F) based on the presence or absence of genes in an 80 kb region consisting of aflatoxin biosynthesis and sugar clusters. In addition, small deletions (<1 kb) or SNPs in other aflatoxin biosynthesis genes also exist (Adhikari *et al.*, 2016). Therefore, relying on the presence or absence of a functional *aflR* gene would classify several atoxigenic strains

as toxigenic inaccurately because their deletion or defect would be located in other aflatoxin biosynthesis genes.

The *aflR* genes could have already changed although they have been amplified, since the mutation of *aflR* only consists of several bases and does not particularly influence the amplification. In addition, there could be other relationships between *aflR* and other genes, which were not studied here. In our future studies, we could expand the screening range and verify the presence or absence of other regulatory genes involved in aflatoxin biosynthesis.

In the present study, strains identified as *A. flavus* and *A. parasiticus* were tested for their aflatoxin producing ability based on solid-state fermentation in soybean culture. Natural substrates, such as cereal grains have been demonstrated to be appropriate substrates for aflatoxin production in laboratory studies with better results (Gourama and Bullerman, 1995; Moreno Romo and Suárez Fernández, 1986). *A. flavus* only produce AFB₁ and AFB₂ and the production of AFB₁ was much greater than that of AFB₂. These results are consistent with those of other studies (Baquião *et al.*, 2013; Magnoli *et al.*, 1998). Ehrlich *et al.* (2004) revealed that that portions of the *cypA* gene were lost in *A. flavus* which was predicted to encode a cytochrome P450 monooxygenase needed in the process of oxidation from B aflatoxins to G aflatoxins. Thus *A. flavus* cannot produce G aflatoxins. The two *A. parasiticus* could produce AFB₁, AFB₂, AFG₁ and AFG₂. Notably, the two isolates produced more AFG₁ than AFB₁. The results are consistent with those of previous studies by Baquião *et al.* (2013) and Peromingo *et al.* (2016) who observed that AFG₁ amounts were always higher than AFB₁ amounts under varying temperature and water conditions.

The aflatoxin biosynthesis gene cluster has been explored previously (Jiujiang *et al.*, 2004). The structural genes are regulated by the transcription factor *aflR* and an accessory regulatory gene, *aflS* (Chang, 2003). Temperature and water are the key factors influencing AFB₁ and AFG₁ biosynthesis. According to the study, temperature changes were responsible for the differences between AFB₁ and AFG₁. Moreover, according to the study, *A. parasiticus* produced higher AFG₁ concentrations at moderate temperatures (20–30 °C), whereas AFB₁ was produced at high temperatures (>30 °C) (Schmidt-Heydt *et al.*, 2010).

Aspergillus strains can produce sclerotia on the CZ medium. In the present study, 125 (80.1%) out of 156 *A. flavus* isolates produced sclerotia after incubation on CZ agar for 14 days, whereas none of the *A. parasiticus*, *A. oryzae* and *A. sojae* isolates produced any sclerotia. No absolute relationship was perceived between sclerotia and aflatoxin production capacity (Giorni *et al.*, 2007). Aflatoxigenic and non-aflatoxigenic *A. flavus* strains similarly produced sclerotia at similar levels (80 vs 81%); however, sclerotia-

producing *A. flavus* strains seemed to be able to produce higher quantities of aflatoxins.

In this study, after the completion of screening, non-aflatoxigenic and aflatoxigenic *A. flavus* were cultured on plate confrontation and soybean culture medium. Indeed, the growth rate and toxin production of toxigenic *A. flavus* strains on confrontation cultures were inhibited. The mechanism of this inhibition has also been described in previous studies. Toxigenic and non-toxigenic *A. flavus* need the same nutrients for growth and reproduction, and there is competition between them. Therefore, a more rapid growth rate of fungal hyphae can ensure that non-toxigenic *A. flavus* strains rapidly occupy the ecological niche, compete for nutrition and grab the living space of toxigenic *A. flavus* to achieve the desired inhibition. Through repeated biological control applications, Weaver and Abbas (2019) tested the hypotheses that applied biocontrol strains competitively displace the indigenous toxigenic strains.

The adaptability of microbial agents to the environment, such as grain types, climate and water, is very important for the biological control effect. Therefore, non-toxigenic *A. flavus* strains screened locally often have more advantages (Moore *et al.*, 2009; Mwakinyali *et al.*, 2019). Non-aflatoxigenic *A. flavus* showed good inhibiting effects on toxigenic *A. flavus* in the mycelial growth and aflatoxin production experiment. Next, a field experiment should be conducted in order to test applicability of the atoxigenic strains.

5. Conclusions

A PCR-RFLP combined with morphological methods was developed for the screening of non-toxigenic *A. flavus* from soils. The aflatoxin producing capacities of the strains were verified using solid-state fermentation experiments. Although some non-toxigenic *A. flavus* strains could have been missed, it could be verified that the isolated non-toxigenic strains lost their capacity producing aflatoxins. Non-toxigenic *A. flavus* had a competitive inhibitory effect against the growth of toxigenic *A. flavus* in plate and natural soybean medium.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/WMJ2019.2490>.

Table S1. Information of experimental strains for PCR-RFLP.

Table S2. Restriction analysis of the *aflR* promoters using *NheI*.

Table S3. Restriction analysis of the *aflR* gene using *HincII* and *PvuII*.

Table S4. Relation between average level of mycotoxin and sclerotium production.

Figure S1. Colony colour on the reverse of AFPA plates of different *Aspergillus* species.

Figure S2. The HPLC chromatogram of aflatoxin G₂, G₁, B₂ and B₁ standards.

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Conflict of interest

The authors declare no conflict of interest.

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