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RESEARCH PAPER

Evaluation of Aflatoxin B1 Biosynthesis in *A. flavus* Isolates from Central India and Identification of Atoxigenic Isolates

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Abstract Present study describes the effect of temperature and pH on biosynthesis of aflatoxin B1 (AFB1) to identify atoxigenic (aflatoxin non-producer) isolates of *Aspergillus flavus*. An indigenous detection method for AFB1 was developed using tandem mass spectrometric method. Detection of AFB1 was carried out in positive polarity using triple quadrapole mass spectrometer with electrospray ionization interface in Multiple Reaction Monitoring (MRM) mode. A total of four *A. flavus* isolates were selected for screening of AFB1 biosynthesis at pH 4.0, 5.5, 7.0, and 8.5 at 27°C. Highest AFB1 biosynthesis in MTCC11866 was found at pH 6.5 while in MTCC9367 it was at pH 7.0. On screening AFB1 biosynthesis in *A. flavus* culture at various temperatures a significant suppression in AFB1 biosynthesis was found at 37°C in comparison to 24 and 27°C in MTCC11866 and MTCC9367. AFB1 was not detected in other two *A. flavus* isolates MTCC11580 and MTCC11588 at any temperature and pH tested. AFB1 nonproducing isolates, MTCC11580 and MTCC11588 could be used as potent biocontrol agent. Additionally, present standardized method for AFB1 detection may find its application in qualitative and quantitative analysis of AFB1 contamination in food crop and other products.

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1. Introduction

Aspergillus flavus is ubiquitous in nature, capable of infecting a broad range of host and produces highly toxic metabolites called aflatoxin [1]. Among various types of aflatoxins *viz*. AFB1, AFB2, AFG1 and AFG2; AFB1 has been found to be most common contaminant of major crop such as corn, peanuts, cotton and other pre/post-harvested crops [2,3]. Aflatoxins are genotoxic carcinogen in which AFB1 is the most toxic compound classified in 1994 by the International Agency for Research on Cancer (IARC) [4,5]. Regular consumption of low level of aflatoxin through contaminated food products causes aflatoxicosis which is characterized by jaundice, rapidly developing ascites, hypertension, vomiting, abdominal pain, pulmonary edema, fatty infiltration and necrosis of the liver [6].

Rice (*Oryzae sativa* L.) is the principal crop of India. Certain environmental conditions such as, temperature, unseasonal rain, chronic drought conditions and poor storage facilities influence aflatoxin contamination [7]. Reddy *et al.* [8] surveyed 20 rice growing states of India for *Aspergillus* spp. and aflatoxin content and found that all the rice samples collected from Chhattisgarh and Meghalaya region were heavily contaminated with aflatoxin. Biological control of aflatoxin biosynthesis through atoxigenic strains of *A. flavus* to reduce aflatoxin contamination in preharvest crop is one strategy that has recently gained prominence [9]. In numerous field experiments, particularly with peanut and cotton, significant reduction in aflatoxin contamination in the range of 70 ~ 90% have been observed consistently

by the use of atoxigenic *Aspergillus* strains [10]. Understanding the biology and regulatory mechanism behind the biosynthesis of AFB1 and development of sensitive assay to screen atoxigenic *A. flavus* isolates would be helpful to limit the aflatoxin exposure.

Yu *et al.* [11] identified genes and enzymes associated with the biosynthesis of aflatoxin. Transcription profile of *A. flavus* at 30 and 37°C using RNA sequencing technology revealed 11 out of the 55 transcripts involved in different secondary metabolic pathways were upregulated at 30°C, and these transcripts encodes enzymes that are involved in aflatoxin biosynthesis [12]. In addition, a key regulatory gene, *aflR*, encodes transcription factor which regulates AFB1 biosynthesis has also been reported [12,13]. However, the intermediate steps and other factors can influence the end product of aflatoxin biosynthetic pathway. Aflatoxins, the end product of the pathway, under the influence of temperature and pH were not quantitatively evaluated. Present study describes the development of MRM method for the first time to screen AFB1 through direct injection using tandem mass spectrometry from India and to assess the influence of temperature and pH on AFB1 biosynthesis. The study also resulted in the identification of not detectable AFB1 in *A. flavus* isolates which could be used as a biocontrol agent.

2. Materials and Methods

2.1. *Aspergillus flavus* isolates and inoculum preparation

Pure culture of *A. flavus* (MTCC9367) was procured from microbial type culture collection and gene bank (MTCC), Chandigarh, India in lyophilized form and was revived in sabraud's dextrose agar (SDA) medium (peptone 10.0 g/L, dextrose 40.0 g/L, pH 5.6, agar 15.0 g/L) at 27°C. Other *A. flavus* isolates used in the study viz. MTCC11580, MTCC11588 and MTCC11866 were isolated from soil, red chilli fruits and rice seeds, respectively, and cultured on SDA. All the isolates were sub cultured on the same medium at 30 days interval. Pure cultures were identified through polyphasic approach and submitted to MTTCC and gene bank, Chandigarh, INDIA and their ITS region sequences were submitted to NCBI Gene Bank, Bethesda, Maryland, USA; KC907366.1 (MTCC11580), KC907367.1 (MTCC11588) and KF317635.1 (MTCC11866).

Conidial suspensions of *A. flavus* isolates were prepared from 7 days old culture plates in 1× phosphate buffer saline (PBS) with 0.05% (v/v) sterile polyoxyethylene sorbitan monooleate (Tween 80, Merck, India) solution (PBST). Conidial count of suspension was determined through direct counting using haemocytometer (Neaubaure, Germany) at 400× magnification using Carl Zeiss Axio.A1 Scope compound microscope. Working suspension was prepared by diluting the conidial suspension with PBST to a definitive count of 10^6 conidia/mL. *A. flavus* isolates were preliminary screened using culture plate methods *viz*. yeast extract sucrose (YES), ammonium hydroxide vapour [14] and neutral red desiccated coconut agar (NRDCA) medium [15].

2.2. Culture plate method for aflatoxin production

There are numerous culture methods available for qualitative screening of aflatoxin production but none of them is solely reliable, hence three different culture methods *viz.* ammonium hydroxide vapour, YES agar with β-cyclodextrin [16] and NRDCA medium were applied for screening purpose. *A. flavus* isolates were cultivated in YES (yeast extract 10.0 g/L, sucrose 150.0 g/L, agar 15.0 g/L, pH 6.3), YES + β-cyd and NRDCA medium (coconut milk (25%, v/v), distilled water (75%, v/v), neutral red (0.2%, w/v), pH 4.6) as a single point inoculation using 2.0 μL of conidial suspension (10^6 conidia/mL). Detailed procedure of each method is described below:

2.2.1. Screening in yeast extract sucrose agar

Fungal isolates were screened in YES agar medium supplemented with 3.0% (w/v) β-cyclodextrin (β-cyd, Himedia, India) as a fluorescence enhancer [17]. The β-cyd solution (3.0%, w/v) was filter sterilized (0.2 μm syringe filter) and added to autoclaved medium with a final concentration of 0.3% (w/v). These YES agar plates were aseptically point inoculated at the centre of culture plates with 2.0 μL of working conidial suspension (10^6 conidia/mL) using a micropipette. Plates were incubated in the dark at 27°C for 3 days. The presence or absence of a fluorescence ring in the agar plate surrounding the mycelia, observed under UV light (365 nm), was scored as positive or negative [18].

Effect of temperature on aflatoxin production was also analyzed through agar plate method using YES agar medium with pH adjusted to 6.3 using the same medium and same inoculum volume and incubating the plates at 24, 27, and 37°C for a period of one week. Plates were examined visually for growth and then under the UV light for fluorescence.

2.2.2. Detection of aflatoxin using ammonium hydroxide vapour method

Based on Saito and Machida [14] YES agar was prepared for the rapid detection of aflatoxin. The center of the plates were single point inoculated with 2.0 μL of conidial suspension and plates were incubated for 3 days in the dark at 27°C. After completion of incubation period plates were inverted and 2 ~ 3 drops of concentrated ammonium

hydroxide solution (30%) was placed on the lid of the plates and left for about 5 min. The underside of fungal colony was observed for the colour change. Aflatoxin producing colonies turn plum red colour, while no colour change occurs in the underside of non-aflatoxin producing fungi [14].

2.2.3. Detection of aflatoxin in neutral red desiccated coconut agar medium

Visual detection of aflatoxin was carried out using modified NRDCA medium in which desiccated coconut milk was replaced with commercially available coconut milk (Thai Coconut Milk Lite, product of Dee Thai Perfect Foods Co. Ltd., Thailand) [15]. In the medium NRDCA plates were single point inoculated at the centre and plates were incubated at 27°C for 3 ~ 4 days. Plates which developed pink coloured ring around the mycelia were scored as positive.

2.3. Culture broth for aflatoxin production

Production of AFB1 was accessed in YES broth (yeast extracts 10.0 g/L, sucrose 150.0 g/L, pH 6.5). About 50 mL of YES broth was transferred to 250 mL flask containing 50 mL culture medium were inoculated with 1.0 mL of working conidial suspension (contains 10^6 conidia). Flasks were incubated in the dark in static condition at 24, 27, (considered as control) and 37°C for 7 days.

The effect of pH on AFB1 biosynthesis was carried out through a separate experiment by adjusting the pH of the medium to 4.0, 5.5, 7.0, and 8.5 with 1N HCl and 1N NaOH using a digital pH meter. Each flask was aseptically inoculated with 1.0 mL conidial suspension and incubated at 30°C for 7 days in the dark under static condition. All the experiments were performed in triplet.

2.3.1. Biosynthesis of aflatoxin at different morphological forms

To access the aflatoxin biosynthesis at different morphological forms, *viz.* conidia, germinating conidia and mycelia, 50 mL of YES broth (pH 6.5) was transferred to 250 mL flask and aseptically inoculated with 1.0 mL of conidial suspension (10^6 conidia/mL) and incubated at 27°C at 100 rpm. Broth mediums were inoculated with higher number of conidia (10^6 conidia) for reliable detection of aflatoxin in minor aflatoxin producing isolates of *A. flavus* [19]. The time point for transition of conidia to germinating conidia and then to mycelia was determined by observing the broth containing conidia under compound microscope using haemocytometer after an interval of 30 min till mycelia formation. In the mycelia stage aflatoxin was extracted at one day interval.

2.4. Extraction of aflatoxin

Culture broth was harvested with equal volume of chloroform. The mixture was incubated at room temperature (25°C) for 15 ~ 20 min in a rotary shaker (100 rpm) followed by separation of organic phase using separatory funnel. The chloroform extract was filtered through Whatman no. 1 filter paper and evaporated to dryness at 37°C in hot air oven. The residue was reconstituted in a fixed volume (500 μL) of HPLC grade methanol (Merck, India) and aseptically filtered using membrane filter (0.2 μm).

2.4.1. Optimization of extraction time

To determine the optimum time for extraction, *A. flavus* isolate MTCC11866 was inoculated into YES broth in six sets as previously described. After proper incubation all sets were pooled together and mixed properly followed by redistribution to equalize the concentration of secreted aflatoxin. The broth culture of each flask was extracted with equal volume of chloroform and harvested with an interval of 15 min.

2.5. Preparation of AFB1 standard solution

Stock solution was prepared from standard AFB1 (Sigma-Aldrich, USA) in HPLC grade methanol (Merck, India) with a concentration of 1 mg/mL and stored at 4°C in the dark until use. To prepare a working solution for MS/MS analysis, stock solution of AFB1 was diluted with methanol water (4:6, v/v) to a final concentration of 1 μg/mL.

2.6. Instrumentation and MRM method development

The MS instrument used in the study consists of a gas generator (Peak scientific, USA), triple quadrapole mass spectrometer (API 2000, Applied Biosystems, USA) with an ESI source. MS analysis of AFB1 was performed in the positive mode of ionization with tandem mass spectrometry. Working standard solution of AFB1 was injected in mass spectrometer through direct infusion using a syringe pump (Hamilton, USA) with a constant flow rate of 10 μL/min. Compound dependent and source dependent parameters *viz*. ion spray voltages (IS), nebulizer gas (GS1), drying gas (GS2), curtain gas, ion source temperature, declustering potential (DP), collision energy (CE), cell entrance potential (CEP) and cell exit potential (CXP) were optimized in semi automatic mode with continuously infusing AFB1. Fragmentation potential (FP) and entrance potential (EP) were left at their respective default value.

Standard AFB1 was first scanned using Q1MS in full scan mode followed by the product ion scanning. Two product ions with high intensity along with m/z 313 as their parent ion were selected for final MRM method development and both were further scanned in precursor

ion mode to confirm their parent molecule. The improved MRM method was finally validated by taking the standard AFB1 as a test material and MRM chromatogram was observed for peaks of both Q1/Q3 masses (241/313 and 269/313). All the mass spectrometric data were processed using Analyst 1.5 software (Applied Biosystems, USA).

2.7. Calibration curve

To prepare the calibration curve, AFB1 standard solution was diluted with methanol water (4:6, v/v) resulting in the concentration range of 0.1 ~ 100 ng/mL. Five point calibration curve was plotted at different concentrations. Each point was repeated thrice. The linearity was excellent as evident from high correlation coefficient (r^2) of 0.999.

3. Results and Discussion

3.1. Detection of aflatoxin

Various methods have been developed to screen large number of *A. flavus* isolates for aflatoxin production in time and cost-effective manner using culture plate methods. Yet culture methods are less sensitive and non-specific for different aflatoxin. However, the methods also produce false positive or false negative results but they could be used for screening purpose in combination with other methods.

3.1.1. Screening of aflatoxin in YES agar

Under the long UV light two strains of *A. flavus viz*. MTCC9367 and MTCC11866 produced blue fluorescence. Blue fluorescence surrounding the mycelia indicates the synthesis of aflatoxin [20] while the bright greenish fluorescence might be due to the production of AFG's (AFG1 and/or AFG2). However, the intensity of fluorescence varies with isolates.

3.1.2. Detection of aflatoxin using ammonium hydroxide vapour

None of the tested strains produced pinkish pigmentation on the reverse of colonies/mycelia with the ammonium hydroxide. It might be due to the false negative results or the isolates might not produce any aflatoxin. However, the *A. flavus* strain MTCC9367 reported previously as aflatoxin producing strain [21] did also not show positive response which confirmed the false negative result. Previous reports confers that the ammonium vapour test produces false positive and false negative results [14,22,23]. Hence the isolates were further subjected to NRDCA and YES with cyclodextrin medium which leads to confirmation of toxigenic or atoxigenic nature of the isolates.

3.1.3. Detection of aflatoxin on NRDCA medium

NRDCA medium provides rapid and direct visual determination of aflatoxin production. All the tested strains showed change in colour as a positive result and indicates production of aflatoxin in the respective isolates. We observed that NRDCA medium was more sensitive than the other tested culture plate methods (data not shown).

3.2. Development of a rapid mass spectrometric method

Tandem mass spectrometry provides the high level of reliability in analyte identification. Direct infusion tandem mass spectrometry is compatible to analyze secondary metabolites using soft ionization techniques like ESI in a very short time. In most of the studies, researchers apply some type of cartridge for cleaning up extracts. Utilizing such cartridge may influence the presence and quantity of the compound in the final extract. We intended to analyze AFB1 in crude extract without clean-up and chromatographic separation, to make the method rapid and feasible. Direct infusion MS can be used for targeted analysis of metabolite and metabolite profiling in several distinct ways: metabolite quantification, structure elucidation by tandem MS or screening of metabolites in a complex biological sample [24,25]. We used tandem MS with ESI interface to assess the biosynthesis of AFB1 in *A. flavus* at low (24 and 27°C) and high temperatures (37°C), and at various pH range.

3.2.1. Optimization of mass spectrometric parameters and MRM method development

Reference AFB1 solution was run in the Q1MS scan mode which showed peak of mass to charge ratio (m/z) 313; protonated ion $[M+H]^+$ of AFB1 depicted in Fig. 1A. The m/z 313 was chosen as parent ion and its declustering potential (DP) was optimized (Fig. 1B) followed by analysis of fragmentation products (Fig. 1C). Product ion scan showed two most intense peaks corresponding to m/z 241 and m/z 269 which were selected for further method development. Their parent masses were confirmed through scanning in precursor ion mode which produced a single peak of m/z 313 (Fig. 2) hence confirmed the parent molecule. Optimization of CE, CEP, CXP, IS, GS1, GS2, DP, FP and EP are shown in Fig. 2.

The optimized values of compound (AFB1) dependent and the source dependent parameters for Q1/Q3 (313/241 and 313/269) are shown in Table 1. MRM mode was adopted, in particular, to increase the sensitivity and specificity of AFB1 detection. Both of the masses (241 and 269, Q3 mass) along with the parent mass (Q1 mass) were taken to develop final MRM method. Considering both the transitions makes the method more sensitive and highly reliable because the method simultaneously analyzes two

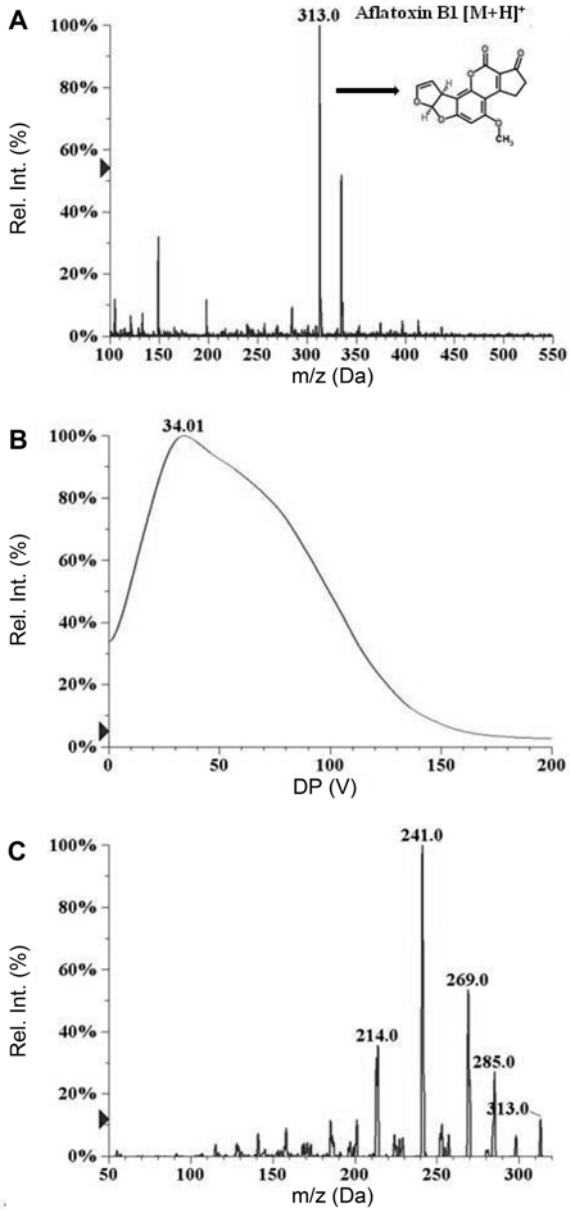

Fig. 1. MS/MS analysis of aflatoxin B1 (m/z 313). (A) Q1MS of standard AFB1 (1.0 µg/mL) showing m/z value of 313 for protonated ion $[M+H]^+$ of AFB1; (B) optimization of DP for AFB1; (C) fragmentation products of AFB1 showing highest intensity of m/z 241 followed by m/z 269.

mass transitions or products of the parent mass (m/z 313 for AFB1). Positive polarity along with ESI source was chosen for analysis in the view of production of abundant protonated molecules $[M+H]^+$ [26]. There were no additional peaks observed from samples of any isolate which showed very high selectivity of MRM mode. Counts per second (CPS) of m/z, 313/241 (Q1/Q3) was applied to calculate the concentrations of AFB1 using a calibration curve (Fig. 3) and expressed in µg/mL of broth culture.

Table 1. Optimized mass spectrometric parameters (source dependent and compound dependent) for Q1/Q3 (m/z) of 313/241 and 313/269 for standard AFB_1

S. no	Parameters	Q1/Q3 (313/241)	Q1/Q3 (313/269)
1	Entrance Potential (EP)	10	10
2	Fragmentation Potential (FP)	400	400
3	Declustering Potential (DP)	34.1	34.1
4	Cell Entrance Potential (CEP)	13.8	12.2
5	Collision Energy (CE)	50.0	42.6
6	Collision cell Exit Potential (CXP)	8.4	9.3
7	Nebulizer Gas (GS1)	40	40
8	Drying Gas (GS2)	50	50
9	Ion source Temperature	350°C	350°C

3.3. Advantage of direct injection mass spectrometry

Aflatoxins have been under study for a long time due to rigorous limit of mycotoxins set by regulatory authorities in various food stuffs [4] which urged for the development of sensitive and rapid technique for easy and confirmed detection. There are advanced techniques described in recent years many of which were based on MS [27]. Such methods require sample purification through column [27] or derivatization [28] for improved detection. Column purification consumes large volume of toxic solvents, time consuming and the column filling material might reduce the quantity of analyte. Present study describes direct injection tandem mass spectrometric method for detection of AFB1. This method excludes column separation or any other purification step without losing sensitivity and specificity. The detection limit of this method was 0.1 ng/g for AFB1 which meets the criteria of European Commission [4] with recovery factor of 70 ~ 90%. After optimization, the analysis can be done rapidly (< 2 min/sample). Although mass spectrometry is a destructive method (sample could not be recovered), a very minute quantity of sample and sensitive detection compensates the requirement of sample recovery. The method could be used for rapid screening of minor aflatoxin producer and atoxigenic and toxigenic isolates due to its high sensitivity and selectivity.

3.4. Optimization of aflatoxin extraction

Culture filtrates were extracted with chloroform at an interval of 15 min. The lowest value of AFB1 was detected in the sample extracted for 15 min while all the other samples extracted for more than 15 min showed significantly higher yield. However, no significant increase in yield was observed in samples extracted for 30 min or more time. These results suggest that the time duration of 30 min is sufficient for extraction of AFB1 using liquid-liquid

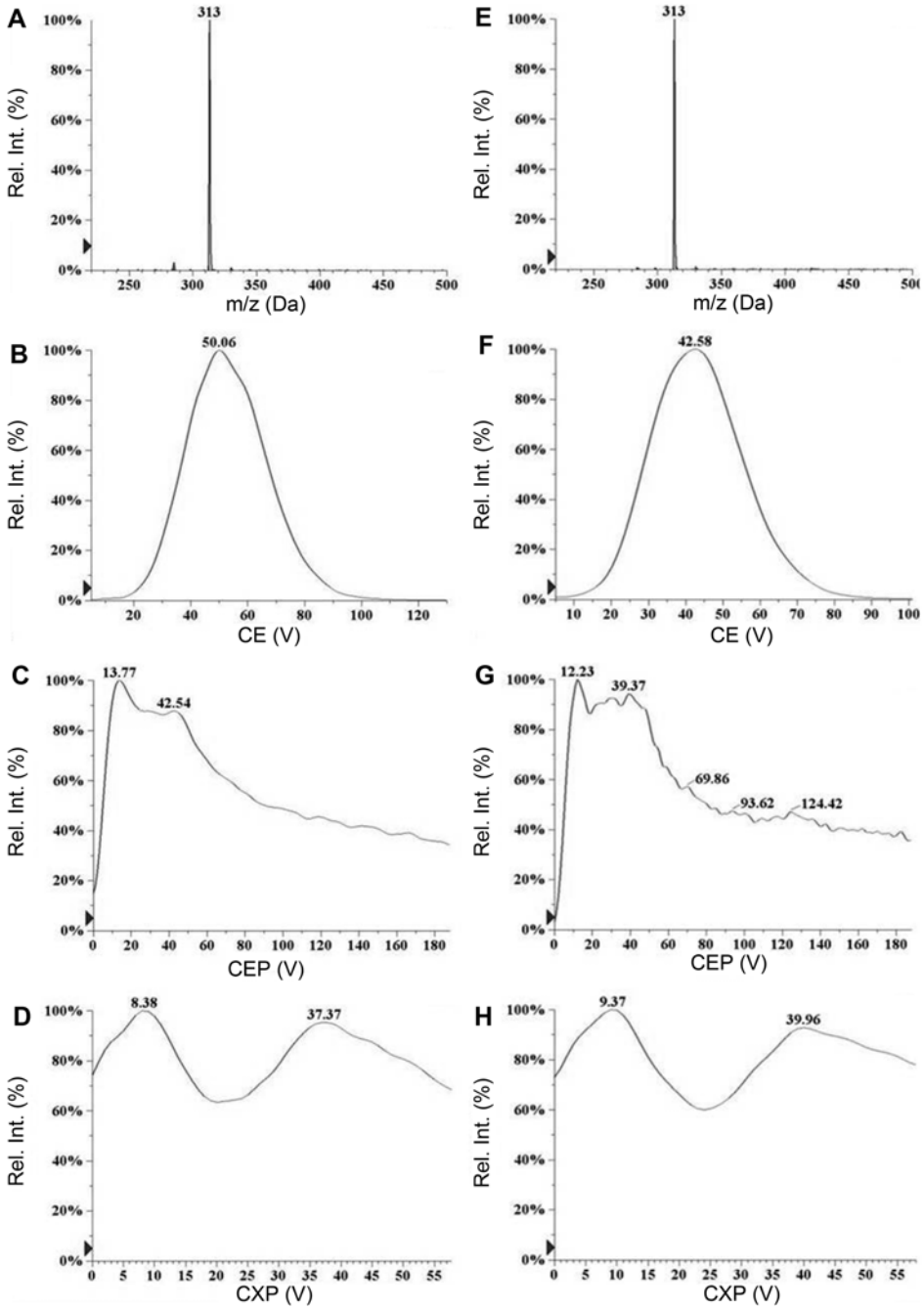

Fig. 2. Optimization of mass spectrometric parameters for m/z 241(A-D) and m/z 269 (E-H): (A) confirmation of parent ion of m/z 241 through precursor ion scan mode; (B) CE of m/z 241; (C) CEP of m/z 241; (D) CXP of m/z 241; (E) confirmation of parent ion of m/z 269 through precursor ion scan mode; (F) CE of m/z 269; (G) CEP of m/z 269; (H) CXP of m/z 269.

extraction with chloroform.

Keeping in mind that aflatoxin production might vary with *A. flavus* isolates, an experiment was set with variable quantity of conidia and estimated aflatoxin production. It was found that aflatoxin production is proportional to conidial concentration. However, a higher number of conidia (10^6 conidia) would be preferable for minor producer to estimate the expression level of aflatoxin and also for reliable detection. Previous reports [19,29] also suggest for similar conidial concentration.

Since the objective was to determine the difference in aflatoxin production at various incubation conditions, therefore, it requires estimation of aflatoxin at a time point where its production occurs in sufficient amount. We found that 7 day incubation is sufficient for AFB1 detection, so we preferred this period for the analysis [19,30].

3.5. Aflatoxin biosynthesis at different morphological forms

AFB1 was not found in conidia and germinating conidial stage in any of the tested *A. flavus* isolates. However, in mycelia stage two of the *A. flavus* isolates, MTCC9367 and MTCC11866, produced significant quantity of AFB1 (2.5 and 25.43 μg/mL for MTCC9367 and MTCC11866, respectively after 5 days of incubation), while in other isolates AFB1 was not detected at any stage. For the first time we report the aflatoxin biosynthesis in different morphological forms through tandem mass spectrometry and confirmed that the aflatoxin production occurs only in the mycelia stage not in conidia or germinating conidia in *A. flavus*. In addition, we also studied the effect of incubation period on aflatoxin biosynthesis. AFB1 biosynthesis was initiated on 4th and 2nd day, with sharply increased on 5th and 3rd day of incubation in *A. flavus* isolate, MTCC9367 and MTCC11866, respectively. Thereafter, a gradual decrease in AFB1 was observed in both the isolates (MTCC9367 and MTCC11866), which might be due to the degradation of accumulated product (Fig. 4).

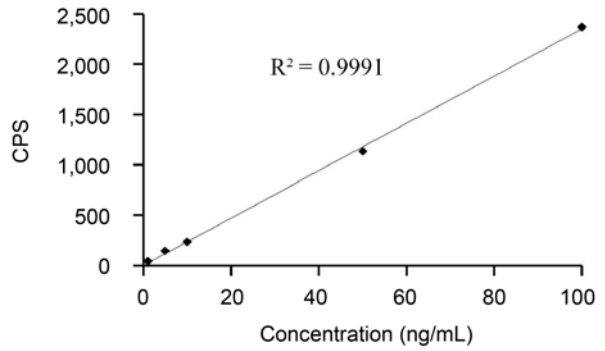

Fig. 3. Calibration curve of AFB1; concentration ranged from 0.1 to 100 ng/mL.

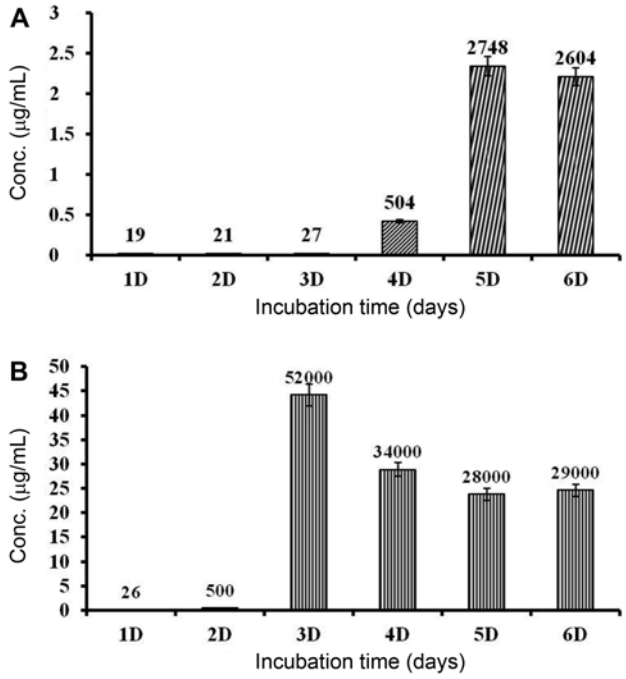

Fig. 4. Biosynthesis profile of AFB1 at different incubation period. Values corresponding to each day are mean of three replicates. Concentration of AFB1 was calculated with the help of the standard curve of AFB1 and cps value of m/z 313/241 (Q1/Q3) at corresponding day. CPS value of each day is displayed over the respective bars. The cps value depicted in the graph is from diluted samples (1/2000). AFB1 biosynthesis in *A. flavus* isolates MTCC9367 (A), in *A. flavus* MTCC11866 (B).

3.6. Influence of temperature on AFB1 biosynthesis

The influence of temperature on aflatoxin (AFB1) production in *A. flavus* showed enhanced AFB1 with increase in temperature from 24°C (2.3×10^4 cps, 19.27 μg/mL) to 27°C (5.1×10^4 cps, 43.63 μg/mL at 27°C) for isolate MTCC11866. However, at higher temperature (37°C) an 8.6 fold decrease (5967 cps, 5.07 μg/mL) was observed as compared to that at 27°C (Fig. 5A). Isolate MTCC9367 showed a similar trend with a significant low level of AFB1 biosynthesis (Fig. 5B), however, the isolate was found more sensitive to temperature as it showed 10 times higher suppression of AFB1 synthesis at 37°C compared to isolate MTCC11866. AFB1 was not detected in *A. flavus*

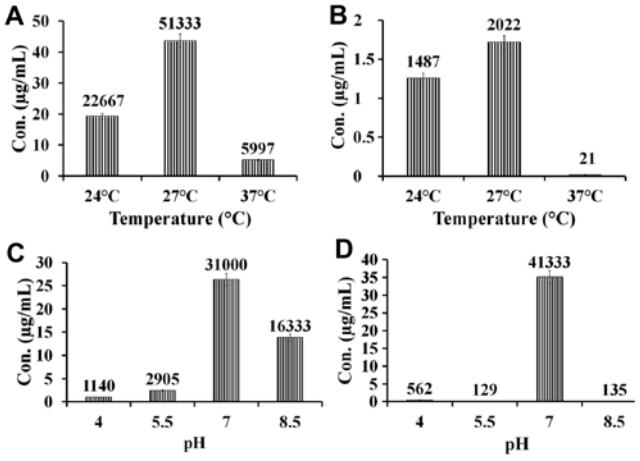

Fig. 5. Effect of temperature and pH on AFB1 biosynthesis in *A. flavus* isolates. *A. flavus* isolates were incubated at different temperatures (24, 27, and 37°C) and pH (4.0, 5.5, 7.0, and 8.5). Values corresponding to each temperature are mean of three replicates. Concentration of AFB1 was calculated with the help of the standard curve and cps value of m/z 313/241 (Q1/Q3) at corresponding temperatures. CPS value of each temperature is displayed over the respective bars. The cps value depicted in the graph is from diluted samples (1/2000). Difference in AFB1 production at various temperatures and *A. flavus* isolate is significant ($P \leq 0.001$). Effect on temperature on *A. flavus* isolates MTCC11866 (A), on *A. flavus* MTCC9367 (B). Effect of pH on *A. flavus* isolates MTCC11866 (C), and *A. flavus* MTCC9367 (D).

isolate MTCC11580 and MTCC11588 at any temperature (24, 27, or 37°C) tested.

The two isolates, MTCC11866 and MTCC9367, showed differential aflatoxin production at different temperatures (Fig. 5), suggesting that AFB1 production varies with *A. flavus* isolates probably due to differential expression of genes associated with AFB1 production. Liu and Chu [31] have reported a 4 folds low expression of *aflR* gene (associated with aflatoxin biosynthesis) at 37°C compared to 29°C in aflatoxigenic *A. parasiticus* strain. Shankar *et al.* [32] reported specific transcripts of *A. fumigatus* in mycelial form at 37°C in order to maintain the infection at human body temperature. Down regulation of key transcripts encoding enzymes involved in aflatoxin biosynthesis, at 37°C in *A. flavus* using RNA sequencing technology has been reported by Yu *et al.* [12]. Though, qualitative and quantitative data support our contention that AFB1 production is temperature dependent. The probable mechanism underlies inhibition of aflatoxin biosynthesis at 37°C or higher temperature in toxigenic *A. flavus* isolates needs detail investigation, specifically the role of heat-shock proteins.

3.7. Influence of pH on AFB1 biosynthesis

On analysing mass spectrometric data of *A. flavus* isolates incubated at different pH, we observed maximum AFB1 biosynthesis at pH 7.0 in MTCC9367 and MTCC11866 (Figs. 5C and 5D). MTCC11866 produced 2.47, 26.35, and 13.88 μg/mL of AFB1 at pH 5.5, pH 7.0 and 8.0, respectively (Fig. 5C). A similar pattern of AFB1 biosynthesis was also observed in isolate MTCC9367 (Fig. 5D). Acidic environment causes more significant reduction in AFB1 synthesis as compared to alkaline one. The data shown in Figs. 5C and 5D indicates a nearly similar trend where AFB1 production was significantly reduced at pH 5.5 (acidic) and at pH 8.0 (alkaline) in all the toxigenic strains tested. Keller *et al.* [33] also reported 5 fold reduced production of mycotoxin in *A. paraciticus* and *A. nidulans* with increasing pH from acidic (pH 4.0) to alkaline pH (pH 8.0). Increasing the initial pH of the medium from 7.0 to 8.5, we found 293 fold decreased biosynthesis of AFB1 in MTCC11866 and about 2 fold in isolate MTCC9367. However, when we considered both experiments (effect of pH and temperature) we found that slightly acidic pH (6.5) greatly increases the AFB1 biosynthesis in MTCC11866. Hence it is clear that the biosynthesis of AFB1 is tightly regulated by pH, although not in the same degree in the two toxigenic isolates (MTCC9367 and MTCC11866) of *A. flavus*. Genes influenced by pH have been well characterized for *Trichoderma virens*, *A. nidulans* and *Fusarium graminearum* [34]. However, further investigation of potential regulatory factors/genes associated with the pH regulatory system and secondary metabolite production in *A. flavus* is deemed require.

3.8. Atoxigenicity of *A. flavus*

Atoxigenic isolates of *A. flavus* contain defective/mutated genes *viz. pksA* [9] or *aflR* [11] which are essential for the biosynthesis of aflatoxin [29]. Atoxigenic strains are currently used to reduce aflatoxin contamination in food crops [9]. The two isolates (MTCC11580 and MTCC11588) used in the present investigation did not show AFB1 biosynthesis at any temperature and pH conditions indicates the possibility of SNP/mutation in *pks* genes, which needs further confirmation by sequencing. The mechanism behind how atoxigenic strains of *A. flavus* suppress the growth of toxigenic strain is not clear. However, it may be hypothesized that the cellular efforts to produce aflatoxin by toxigenic isolates of *A. flavus* are much higher in comparison to atoxigenic *A. flavus* isolates. This possibly allows atoxigenic isolates to surpass the growth over the toxigenic *A. flavus* isolates.

4. Conclusion

In conclusion the optimum parameters for the extraction of AFB1 and physiological factors (temperature and pH) influencing AFB1 biosynthesis in *A. flavus* were established. A rapid and cost effective, direct injection electrospray ionization mass spectrometric (ESI-MS) method has been developed for the rapid detection of AFB1 owing to its high sensitivity and specificity using tandem mass spectrometry. The results obtained in the current work confirmed that the biosynthesis of AFB1 is temperature and pH sensitive. High temperature (37°C) or extreme pH conditions suppresses AFB1 biosynthesis in *A. flavus* as revealed by tandem MS. We also report that amount of biosynthesis of AFB1 varies in toxigenic isolates of *A. flavus*. This is the first report that identifies not detectable AFB1 *A. flavus* isolates from the central region of India, Chhattisgarh. The indigenous atoxigenic isolates of *A. flavus* (MTCC11580 and MTCC11588) could be used as a promising biocontrol agent to reduce aflatoxin contamination in rice.

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References

1. Shankar, J. (2013) An overview of toxins in *Aspergillus* associated with pathogenesis. *Int. J. Life Sc. Bt. Pharm. Res.* 2: 19-31.
2. Georgiadou, M., A. Dimou, and S. Yanniotis (2012) Aflatoxin contamination in pistachio nuts: A farm to storage study. *Food Control* 26: 580-586.
3. Shankar, J., T. Madan, S. F. Basir, and P. U. Sarma (2005) Identification and characterization of polyubiquitin gene from cDNA library of *Aspergillus fumigatus*. *Ind. J. Clin. Biochem.* 20: 208-212.
4. European Commission (2006) Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union L* 364: 5-23.
5. Kensler, T. W., B. D. Roebuck, G. N. Wogan, and J. D. Groopman (2011) Aflatoxin: A 50 year odyssey of mechanistic and translational toxicology. *Toxicol. Sci*. 120: 28-48.
6. Lewis, L., M. Onsongo, H. Njapau, H. Schurz-Rogers, G. Luber, S. Kieszak, J. Nyamongo, L. Backer, A. M. Dahiye, A. Misore, K. DeCock, C. Rubin, and the Kenya Aflatoxicosis Investigation Group (2005) Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environ. Health Persp.* 113: 1763-1767.
7. Atas, M., Y. Yardimci, and A. Temizel (2012) A new approach to aflatoxin detection in chili pepper by machine vision. *Comput. Electron. Agr.* 87: 129-141.
8. Reddy, K. R. N., C. S. Reddy, and K. Muralidharan (2009) Detection of *Aspergillus* spp. and aflatoxin B1 in rice in India. *Food Microbiol.* 26: 27-31.
9. Chang, P. and S. T. Hua (2007) Nonaflatoxigenic *Aspergillus flavus* TX9-8 competitively prevents aflatoxin accumulation by *A. flavus* isolates of large and small sclerotial morphotypes. *Int. J. Food Microbiol.* 114: 275-279.
10. Dorner, J. W. (2008) Management and prevention of mycotoxins in peanuts. *Food Addit. Contam.* 25: 203-208.
11. Yu, J., P. K. Chang, K. C. Ehrlich, J. W. Cary, D. Bhatnagar, T. E. Cleveland, G. A. Payne, J. E. Linz, C. P. Woloshuk, and J. W. Bennett (2004) Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 77: 1253-1262.
12. Yu, J., N. D. Fedorova, B. G. Montalbano, D. Bhatnagar, T. E. Cleveland, J. W. Bennett, and W. C. Nierman (2011) Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA Seq. *FEMS Microbiol. Lett.* 322: 145-149.
13. Levin, R. E. (2012) PCR detection of aflatoxin producing fungi and its limitations. *Int. J. Food Microbiol.* 156: 1-6.
14. Saito, M. and S. Machida (1999) A rapid identification method for aflatoxin producing strains of *A. flavus* and *A. parasiticus* by ammonia vapour. *Mycoscience* 40: 205-211.
15. Atanda, O. O., M. C. Ogunrinu, and F. M. Olorunfemi (2011) A neutral red desiccated coconut agar for rapid detection of aflatoxigenic fungi and visual determination of aflatoxins. *World Mycotoxin J.* 4: 147-155.
16. Davis, N. D., U. L. Diener, and D. W. Eldridge (1966) Production of aflatoxins B1 and G1 by *Aspergillus flavus* in a semisynthetic medium. *J. Appl. Microbiol.* 14: 378-389.
17. Vázquez, M. L., A. Cepeda, P. Prognon, G. Mahuzier, and J. Blais (1991) Cyclodextrins as modifiers of the luminescence characteristics of aflatoxins. *Anal. Chim. Acta* 255: 343-350.
18. Dyer, S. K. and S. Mc Cammon (1994) Detection of toxigenic isolates of *Aspergillus flavus* and related species on coconut cream agar. *J. Appl. Bacteriol.* 76: 75-78.
19. Probst, C. and P. J. Cotty (2012) Relationships between *in vivo* and *in vitro* aflatoxin production: reliable prediction of fungal ability to contaminate maize with aflatoxins. *Fungal Biol.* 116: 503-510.
20. Fente, C. A., O. J. Jaimez, B. I. Vázquez, C. M. Franco, and A. Cepeda (2001) New additive for culture media for rapid identification of aflatoxin producing *Aspergillus* strains. *Appl. Environ. Microbiol.* 67: 4858-4862.
21. Anand, R., J. Shankar, A. P. Singh, and B. N. Tiwary (2013) Cytokine milieu in renal cavities of immunocompetent mice in response to intravenous challenge of *Aspergillus flavus* leading to aspergillosis. *Cytokine* 61: 63-70.
22. Abbas, H. K., R. M. Zablotowicz, M. A. Weaver, B. W. Horn, W. Xie, and W. T. Shier (2004) Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. *Can. J. Microbiol.* 50: 193-199.
23. Kumar, S., M. Shekhar, K. A. Ali, and P. Sharma (2007) A rapid technique for detection of toxigenic and non-toxigenic strain of *Aspergillus flavus* from maize grain. *Ind. Phytopathol.* 60: 31-34.
24. Kuzma, M., A. Jegorov, P. Kačer, and V. Havlíček (2001) Sequencing of new beauverolides by high performance liquid chromatography and mass spectrometry. *J. Mass Spectrom.* 36: 1108-1115.
25. Villas-Bôas, S. G., S. Mas, M. A. Kesson, J. Smedsgaard, and J. Nielsen (2005) Mass spectrometry in metabolome analysis. *Mass Spectrom. Rev.* 24: 613-646.
26. Spanjer, M. C., P. M. Rensen, and J. M. Scholten (2008) LC-MS/MS multi method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess*. 25: 472-489.
27. Wang, L., Z. Wang, W. Gao, J. Chen, M. Yang, Y. Kuang, L. Huang, and S. Chen (2013) Simultaneous determination of aflatoxin B1 and ochratoxin A in Licorice roots and fritillary bulbs by solid-phase extraction coupled with high-performance liquid chromatography-tandem mass spectrometry. *Food Chem.* 138: 1048-1054.
28. Gerbaldo, G. A., C. M. Pereyra, L. R. Cavaglieri, F. Ruiz, L. Pascual, A. M. Dalcero, and I. L. Barberis (2011) Surveillance of aflatoxin and microbiota related to brewer's grain destined for swine feed in Argentina. Vet. Med. Int. DOI:10.4061/2011/912480.
29. Ouattara-Sourabie, P. B., P. A. Nikiema, N. Barro, A. Savadogo, and A. S. Traore (2012) Aflatoxigenic potential of *Aspergillus* spp. isolated from groundnut seeds, in Burkina Faso, West Africa. *Afr. J. Microbiol. Res.* 6: 2603-2609.
30. Reverberi, M., M. Punelli, C. A. Smith, S. Zjalic, M. Scarpari, V. Scala, G. Cardinali, N. Aspite, F. Pinzari, G. A. Payne, A. A. Fabbri, and C. Fanelli (2012) How peroxisomes affect aflatoxin biosynthesis in *Aspergillus flavus*. *PLoS ONE* 7: e48097. doi:10.1371/journal.pone.0048097.
31. Liu, B. H. and F. S. Chu (1998) Regulation of *aflR* and its product, AflR, associated with aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 64: 3718-3723.
32. Shankar, J., S. Nigam, S. Saxena, T. Madan, and P. U. Sarma (2004) Identification and assignment of function to the genes of *Aspergillus fumigatus* expressed at 37°C. *J. Eukaryot. Microbiol.* 51: 428-432.
33. Keller, N. P., C. Nesbitt, B. Sarr, T. D. Phillips, and G. B. Burow (1997) pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. *Phytopathol.* 87: 643-648.
34. Trushina, N., M. Levin, P. K. Mukherjee, and B. A. Horwitz (2013) Pac C and pH-dependent transcriptome of the mycotrophic fungus *Trichoderma virens*. *Genomics* 14: 138.