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Expression of *aflR*, *veA* and *laeA* as regulators of aflatoxins and cyclopiazonic acid biosynthesis pathway in *Aspergillus flavus*

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ABSTRACT

In this study, the production of aflatoxin B_1 (AFB_1) and cyclopiazonic acid (CPA) was investigated in toxigenic and non-toxigenic *Aspergillus flavus* with respect to expression of *aflR*, *veA* and *laeA* genes that are involved in toxin production. *A. flavus* strains were cultured in YES broth at 28 °C for 4 days and AFB_1 and CPA were measured by high performance liquid chromatography (HPLC). The expression of *aflR*, *veA* and *laeA* was assessed in toxigenic and non-toxigenic strains by Real-Time PCR. The results showed that the highest concentrations of AFB_1 and CPA were 9450.56 and 403.85µg/g fungal dry weight, respectively. *A. flavus* isolates were AFB_1 and CPA producer (9450.56 and 377.52 µg/g; chemotype I), AFB_1 producer (2024.80 µg/g; chemotype II), CPA producer (403.85 µg/g; chemotype III), and non-producer (chemotype IV). The results of the analysis of *aflR*, *veA* and *laeA* gene expression between toxigenic and non-toxigenic *A. flavus* isolates did not show any significant correlation between the expression of these genes and AFB_1 and CPA production. Since the incidence of AFB_1 and CPA producing *Aspergillus* in the environment is important from the view point of public health, further evaluation of distribution and expression of different genes of aflatoxins biosynthetic pathway in *A. flavus* strains is recommended in a larger population from different geographic locations of Iran.

1. Introduction

Mycotoxins are toxic secondary metabolites which considered as important contaminants of the food, feed and agricultural commodities. Toxigenic *Aspergillus* strains have been reported from a wide array of agricultural crops. Although hey are distributed in soil, air and decaying materials, agricultural soil serves as the main reservoir of these fungi all over the world (Razzaghi-Abyaneh et al., 2006; Sepahvand et al., 2011; Sepahvand et al., 2013; Amani et al., 2012; Razzaghi-Abyaneh et al., 2014). Surveys of *Aspergillus* section *Flavi* revealed that the diversity and proportions of isolates from different geographical regions which are able to produce different types of aflatoxins at various levels may be quite different (Razzaghi-Abyaneh et al., 2006; Sepahvand et al., 2011; Jamali et al., 2012). It has been proven that *A. flavus* has a wider distribution than that of both *A. parasiticus* and *A. nomius* in soil samples regardless the geographic source of soils (Vaamonde et al., 2003).

Aflatoxins (AFs), a group of polyketide-derived furanocoumarins, are the most toxic and

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carcinogenic compounds which together with cyclopiazonic acid (CPA), an indole-tetramic acid are produced by the ubiquitous genera of molds *Aspergillus* and *Penicillium* (Chang et al., 2009). AFB_1 and CPA are important natural contaminants with real significance for public health (Razzaghi-Abyaneh et al., 2014; Sepahvand et al., 2013). CPA mainly produces by some species of the genera *Aspergillus* and *Penicillium*. Like aflatoxins, the benefit of CPA to the producing fungi is not clear.

The most important aflatoxin producers are members of the *Aspergillus* section *Flavi*, particularly *A. flavus*, *A. parasiticus*, and *A. nomius*. Since the original finding of CPA production by *A. flavus*, the relationship between CPA and aflatoxin production (only B-type) by *A. flavus* has been investigated (Chang et al., 2009; Amani et al., 2012). Enzymes and the regulatory proteins involved in AF biosynthetic pathway in *A. flavus* are encoded by more than 25 genes in a 75-kb cluster (Unnevehr and Grace 2013). The expression and correlation of *aflR* and *aflQ* was reported to be significant in *A. parasiticus* as determined by RT-PCR (Sweeney et al., 2000; Jamali et al., 2012). *LaeA* is proven to have a key role on *A. flavus* secondary metabolism where the fungus produces various beneficial and harmful metabolites including the carcinogen aflatoxins (Kale et al., 2008; Bok and Keller, 2004; Bok et al., 2006). *VeA* is another important regulator of *A. flavus* secondary metabolites and sclerotia (Kale et al., 2008; Stinnett et al., 2007). *VeA* is necessary for aflatoxin production and *aflR* expression in *A. parasiticus* (Calvo et al., 2004) and *A. flavus* (Duran et al., 2007). Although aflatoxin biosynthesis pathway is a best known pathway of secondary metabolism initiated by hexanoate synthesis from acetate that is catalyzed by a polyketide synthase (PKS), very little has been documented about CPA formation. Only recently, three clustered biosynthetic genes of CPA production in *Aspergillus flavus* and closely related *A. oryzae* has been identified (Georgianna et al., 2009). Despite established role of *aflR*, *veA* and *laeA* as direct and indirect regulator of aflatoxin biosynthesis, there is little information about the exact function of these genes in concurrent aflatoxin and CPA formation.

This study aims to evaluate correlation between mycotoxin producing ability in toxigenic and non-toxigenic *A. flavus* strains isolated from peanuts soil samples with the expression pattern of *aflR*, *veA* and *laeA* genes as regulators of AFB_1 and CPA biosynthesis pathway.

2. Materials and Methods

2.1. Strain and culture conditions

Toxigenic *A. flavus* CMI 102566, and non-toxigenic *A. flavus* CMI 93803 were obtained from Pathogenic Fungi Culture Collection (PFCC), Pasteur Institute of Iran (http://en.pasteur.ac.ir/pages.aspx?id=586).
These fungi and two strains of AFB_1 and CPA producers isolated from pistachio soils from our previous study were cultured on Sabouraud Dextrose Agar (E. Merck, Germany) medium for 7 days at 28°C. For monitoring the AFB_1 and CPA production, the spores were harvested by adding sterile water containing 0.01 % Tween 80 and were then inoculated into 250 Erlenmeyer flasks containing 100 mL Yeast Extract Sucrose broth (yeast extract 2 % and sucrose 20 %). The flasks were incubated for 3 days at 28°C. Two separate experiments were carried out in two triplicate sets each.

2.2. Detection of AFB_1 and CPA production by TLC (Thin Layer Chromatography)

After culturing all tested *A. flavus* strains, the mycelia was separated from the culture medium using centrifugation and AFB_1 and CPA were extracted from the culture broth by chloroform extraction. The extracts of the culture broth and the standard of AFB_1 and CPA (Sigma-Aldrich) were spotted on TLC silica gel $60F_{254}$ plates, developed by chloroform- ethyl acetate- formic acid 90% (6:3:1, v/v/v) and toluene–ethyl acetate–formic acid (5:4:1, v/v/v) as mobile phase, respectively. AFB_1 was visualized under UV light (365 nm) and photographed with a TLC scanner (CAMAG, Switzerland). CPA was visualized under spraying with Erlich reagent (1 g p-dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml concentrated HCl) as purple spot with R_f equal to 0.68 (Amani et al., 2012).

2.3. Quantitation of AFB_1 and CPA by HPLC (High Performance Liquid Chromatography)

For quantitation of AFB_1 and CPA, chloroformic extract of the culture broth was injected into a HPLC (KNAUER D-14163 UV–VIS system, Berlin, Germany) according to Razzaghi-Abyaneh et al. 2007. Briefly, 50 µl of each chloroformic extracts were injected into the HPLC column (TSK gel ODS-80TS; 4.6 mm ID × 15.0 cm, TOSOH BIOSCIENCE, Japan) and eluted at a flow rate of 1 ml/min by water/acetonitrile/methanol (60:25:15, v/v/v) for AFB_1 and by methanol/water (70:30) contained 300 mg $ZnSO_4 \cdot 7H_2O$ for CPA. The elution time of the samples was compared with pure AFB_1 and CPA standards and quantified on the basis of the ratio of the peak area of samples to those of the standards. The amounts of AFB_1 and CPA were measured at wavelengths of 365 and 284 nm, respectively.

2.4. Fungal dry weight determination

All contents of each well including the culture media and the fungal biomass were filtered through a thin layer of cheese cloth and were thoroughly washed with distilled water. A known weight of mycelia was placed in a stainless steel container and allowed to dry at 80 °C till a constant weight was obtained (Razzaghi-Abyaneh et al., 2008). The net dry weight of the mycelia was then determined.

2.5. Evaluation of aflR, veA and laeA genes expression on AFB_1 and CPA production

RNA extraction and cDNA preparation: After culturing all tested *A. flavus* strains for 3 days at 28 °C, the harvested mycelia mass was flash-frozen in liquid nitrogen and grounded to a fine powder in a porcelain mortar. Total RNA was purified from the homogenized fungal mycelia using TRIZOL reagent, treated with RNase-free DNase (Fermentas). First-strands cDNA were prepared from a total of 1000 ng RNA, using Revert Aid M-MuLV reverse transcriptase with random hexamer primers, according to manufacturer's instructions (Fermentas cDNA kit) (Jahanshiri et al., 2012).

Real-time PCR analysis: Real-time quantitative RT-PCR was carried out using SYBR green master mix (Applied Biosystems) in a final volume of 25 µL for each reaction by ABI PRISM 7500 thermal cycler (Applied Biosystems). The primer sets (Table 1) in a two-step PCR conditions were used with an initial incubation at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min (Jahanshiri et al., 2015). The reaction set also included negative and RT controls (water and RNA instead of the samples, respectively). Each experiment was repeated three times. Agarose gel electrophoreses was performed to confirm the correct size of amplicons and the absence of non-specific bands. The relative quantification of a target gene in comparison with a reference gene was calculated using Real-Time PCR efficiencies from the given slopes in Light Cycler software. The specificity of the reactions was checked by analyzing the melt curves, which display a single sharp peak. The corresponding Real-time PCR efficiency (*E*) of one cycle in the exponential phase was calculated according to the equation:
$E = 10[-1/\text{slope}]$. PCR results were analyzed by relative quantification, using β-actin expression as the reference gene. ΔCt was calculated using the following formula: [ΔCT = CT (target) − CT (reference)]. The gene expression levels were determined by $2^{-\Delta Ct}$ method. The fold increases (FI) were calculated using the comparative threshold method ($2^{-\Delta\Delta CT}$).

2.6. Statistical analysis

The results were analyzed by SPSS version 16 program for Windows (SPSS Inc., Chicago, IL). Analyses of variance (ANOVA) and Tukey's multiple comparison tests at 5 % significance level were used to compare the means of fungal incidence and toxin production data. $P < 0.05$ was considered significant.

Table 1. Primer sequences and their PCR products

Gene	Forward	Reverse	Product size (bp)	Ass. No
veA	ACTTGGACCGCCCATCTCAAGC	CTTCATGCCGCACGGAAAGATC	179	DQ296645.11
laeA	GCTGGTACAATTTGGCTGTC	CGCCTCCGACTTGACTTCTG	158	XM_002374798.1
aflR	GGCTGGTCAGGAGCAAAGC	CCCCGAATTCCGAATCG	189	FN398162.1
β-actin	TCCCTGGAGAAGAGCTACG	GTAGTTTCGTGGATGCCACA	176	X16377

3. Results

The presence of AFB_1 and CPA in *A. flavus* strains was shown in Fig 1 by TLC. The blue and purple lines indicated the presence of AFB_1 and CPA (a, b respectively). As shown in Table 2, *A. flavus* strains were classified into 4 chemotypes based on the ability for producing mycotoxins. The strains capable to produce AFB_1 and CPA (9450.56 and 377.52 µg/g) were classified as chemotype I. AFB_1 producer (2024.80 µg/g; chemotype II), CPA producer (403.85 µg/g; chemotype III), and non-mycotoxins producer (chemotype IV) were classified as remained chemotype II- IV, respectively. The quality and measure of extracted RNA and cDNA from strains were confirmed by reading the ratio and electrophoresis on agarose gel (1%) (Fig. 2).

The production of AFB_1 and CPA was studied at the mRNA level. *β-actin* gene exhibited no changes in expression between toxigenic and non- toxigenic fungi and was used as the reference gene for data analysis. There was not seen any difference at transcriptional level of *aflR*, *veA* and *laeA* between all tested isolates. As depicted in Fig. 3, , the highest expression of *aflR*, *veA* and *laeA* was compared in toxigenic and non-toxigenic *A. flavus* strains. Data are means ± SD (n=6) of two separate experiments in triplicate sets each ($P< 0.05$). Based on the statistical analyses, the reduction in the expression of aflatoxin biosynthesis genes was no significant for all genes examined in this study.

4. Discussion

In the present study, correlation of gene expression in toxigenic and nontoxigenic *Aspergillus flavus* was evaluated in relation to the production of AFB_1 and CPA (Georgianna et al., 2009). The CPA and aflatoxin gene clusters are resided in chromosome 3 in the *A. flavus* genomes (Chang et al., 2009). It has been documented that at least three genes involved in CPA synthesis are correlate well with current as well as previous studies of the biosynthesis of this indole-tetramic acid product (Chang et al., 2009). Although CPA is not a potent acute toxin and few incidents of mycotoxicoses have been reported, aflatoxin B_1 is a potent carcinogen. Little has been documented about the synergistic effects of the two toxins so, more research is needed. With respect to the co-localization of the two gene clusters, it is known why strains of *A. flavus* have different abilities to produce aflatoxin and CPA (Chang et al., 2009).

A meaningful reduction of aflatoxin production was showed after loss of expression of the *aflR* in a *A. flavus ΔlaeA* strain in comparison to the wild type. It has been shown that LaeA activity provides directed expression of many of aflatoxin biosynthesis genes with loss of *laeA* (Bok et al., 2006; Perrin et al., 2007). Toxigenic *A. flavus* isolates produce only B-type aflatoxins and CPA, while *A. parasiticus* strains always produce B- and G-type aflatoxins but not CPA (Chang et al., 2009). As evidenced in several studies, the ability of *A. flavus* isolates to produce CPA is strain dependent. The incidence of CPA production by *A. flavus* is high (>70%) in Argentina and in the United States (Vaamonde et al., 2003).

Table 2. Chemotype patterns of *A. flavus* strains based on aflatoxin B_1, and CPA production

***A. flavus* strain**	**Fungal dry weight (mg)**	**CPA (µg/g DW)**	**AFB_1 (µg/g DW)**	**Chemotype**
CMI 102566	39.9 ± 3.3	377.5 ± 29.7	9450.6 ± 892.4	I
PICC-AF98	44.5 ± 6.6	0.00	2024.8 ± 179.3	II
PICC-AF69	46.9 ± 4.8	403.8 ± 55.3	0.00	III
CMI 93803	35.6 ± 6.0	0.00	0.00	IV

Results are the means ± SD of two separate experiments in triplicate sets. Dry weight; DW

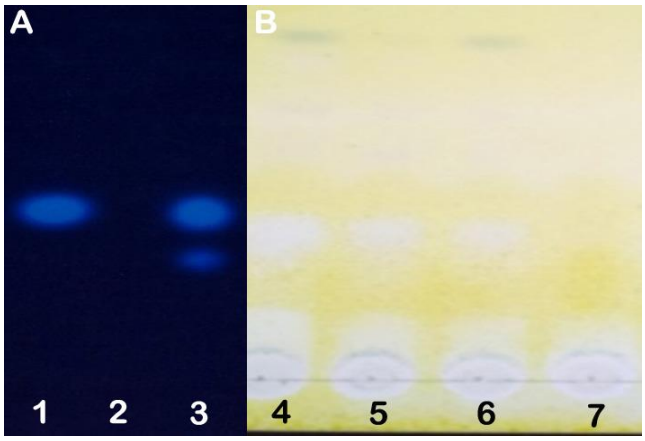

Figure 1. Thin-layer chromatography analysis of AFB^1 (A) and CPA (B) production in *A. flavus* strains: 1A) AFB^1 standard, 2A) non-toxigenic *A. flavus* PICC-AF69, 3A) toxigenic *A. flavus* PICC-AF98, 4B) CPA standard, 6B) toxigenic *A. flavus* PICC-AF69, 5B & 7B) non-toxigenic *A. flavus* PICC-AF98 and *A. flavus* CMI 93803.

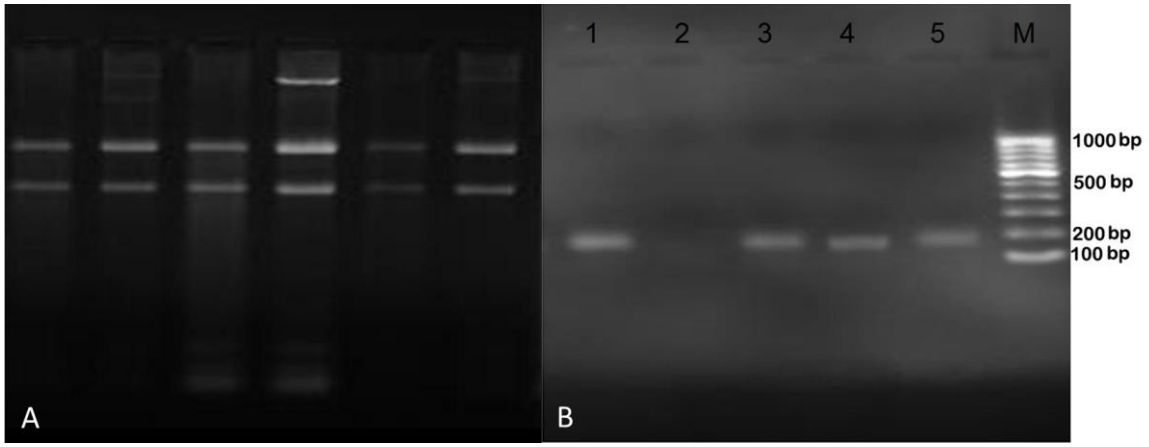

Figure 2. A) The RNA extraction was compared between toxigenic and non-toxigenic strains and B) cDNA preparation of *aflR*, *veA* and *laeA* shown in *A. flavus* CMI 102566. M; DNA ladder 100bp, lanes 1; *β-actin*, 2; negative control, 3; *veA*, 4; *laeA*, 5; *aflR*.

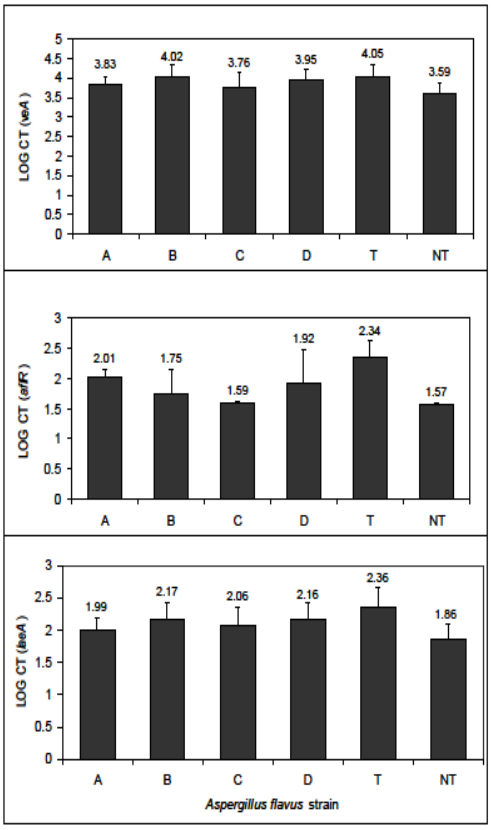

Figure 3. The expression of *aflR*, *veA* and *laeA* was compared in toxigenic and non-toxigenic *A. flavus* strains. A; (AFB_1 and CPA producer), B; (AFB_1 producer), C; (CPA producer), D; non-producer, T; (toxigenic *A. flavus* CMI 102566), and NT (non-toxigenic *A. flavus* CMI 93803). Data are means ± SD (n=6) of two separate experiments in triplicate sets each ($P< 0.05$)

The data from our previous study clearly showed that diverse populations of CPA- and AF-producing *A. flavus* strains are present in peanut field soils. These fungi have considerable variation in the types and amounts of mycotoxins produced (Amani et al., 2012). Jahanshiri et al. reported that the expression of *ver-1*, *nor-1*, *pksA*, *omtA* and *aflR* genes involved in *A. parasiticus* NRRL2999 aflatoxin biosynthetic pathway can affect aflatoxin production (Jahanshiri et al., 2012; Jahanshiri et al., 2015). Razzaghi-Abyaneh et al. reported that dillapiol isolated from the essential oil of dill inhibited AFG_1 production by *Aspergillus parasiticus*. The authors suggested that a possible inhibitory mechanism of dillapiol on AFG_1 production was on the activity of a cytochrome P450-dependent enzyme. Since dillapiol did not inhibit the production of AFB_1, dillapiol may selectively inhibit CypA or the unknown enzyme to stop the pathways from OMST to AFG_1 (Razzaghi-Abyaneh et al., 2007).

Although there are huge data about aflatoxin biosynthesis pathway in producing fungi, this pathway is one of the most complex pathways of secondary metabolism in fungi (Satterlee et al., 2016). Several studies were provided evidences for obvious roles of LaeA and VeA in the development and pathogenesis of *A. flavus* (Duran et al., 2007; Kale et al., 2008). The loss of both genes blocks the production of sclerotia and aflatoxins. Using RT-PCR, it has been shown that inhibition of the transcription of *pksA*, *ver-1* and *omtA* significantly suppresses the pathway regulatory gene, *aflR*. It has been shown that *A. flavus* strains unable to form AFs, due to deletions in AF gene cluster are usually fail to produce CPA (Chang et al., 2009). Production of CPA requires the presence of the *veA* gene. Furthermore, it is found that some other genes like as *rmtA* control the expression of the *veA*, suggesting *rmtA* regulatory system is functionally connected with *veA* (Satterlee et al., 2016). It is possible that the epigenetic mechanism involving genes effect the

production of fungal metabolites.

In the present study, we focused on the expression of the global regulatory gene *laeA*, the developmental regulatory gene, *veA*, and the aflatoxin biosynthetic pathway specific regulatory genes, *aflR*. Our results indicated that the analysis of gene expression of *aflR*, *veA* and *laeA* between toxigenic and non-toxigenic *A. flavus* isolates with respect to the production of AFB_1 and CPA did not show any significant correlation between the expression of these genes and AFB_1 and CPA production among the examined strains. These findings further substanciate the importance of gene profiling and expression in *Aspergillus* section *Flavi* and could contribute to set the bases of novel control strategies to reduce the negative impact caused by *A. flavus* and other detrimental fungal species. Since the incidence of AFB_1 and CPA producing *Aspergillus* in the environment is important from the view point of public health, further evaluation of distribution and expression of different genes of aflatoxins biosynthetic pathway in *A. flavus* strains is recommended in a larger population from different geographic locations of Iran.

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Refereces

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