ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 5, pp. 642–646. © Pleiades Publishing, Ltd., 2013.

EXPERIMENTAL ARTICLES

Molecular Differentiation between Aflatoxinogenic and Non-Aflatoxinogenic Strains of *Aspergillus flavus* and *Aspergillus parasiticus*[1]

H. Moubasher[2], A. Abu Taleb, and H. H. Senousy

Botany Department, Faculty of Science, Cairo University, Giza, Egypt

Received December 28, 2012

Abstract—Three types of media and a multiplex PCR procedure with a set of four primers were used to differentiate between aflatoxinogenic and non-aflatoxinogenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Four sets of primers were the *aflR*, *nor-1*, *ver-1*, and *omt-A* genes of the aflatoxin biosynthetic pathway. Multiplex PCR showed that the four aflatoxinogenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the aflatoxin biosynthetic pathway which encode for the products. Non-aflatoxinogenic strains gave varying results with two, three, or four banding patterns. A banding pattern in seven non-aflatoxinogenic strains resulted in non-differentiation between these and aflatoxinogenic strains.

Keywords: aflatoxinogenic, non-aflatoxinogenic, *Aspergillus flavus*, *Aspergillus parasiticus*, PCR, *aflR*, norsolonic acid, α-ketoreductase and *O*-methyltransferase A genes

DOI: 10.1134/S0026261713050160

Production of carcinogenic aflatoxins has been reported for members of *Aspergillus* section *Flavi*, *Aspergillus* section *Nidulantes* and a newly proposed *Aspergillus* section *Ochraceorosei* [1]. Aflatoxins are toxic and carcinogenic polyketide-derived toxins that frequently contaminate agricultural commodities both pre- and postharvest leading to significant agronomic losses and serious health threats to both humans and domestic animals [2, 3]. The most important aflatoxin producers in both medical and economic aspects are *Aspergillus flavus* and *Aspergillus parasiticus* [4].

There are only four major aflatoxins, B_1, B_2, G_1, and G_2, which are important natural contaminants of crops and agricultural commodities. Among the naturally occurring aflatoxins (AFs), aflatoxin B_1 is considered the most potent hepatotoxic and hepatocarcinogenic agent. Aflatoxins have been detected in numerous agricultural commodities, such as corn, peanuts, cereal grains, whole wheat, rye breads, oil seeds, cottonseed, and tree nuts [5].

The ability of *A. flavus* strains to produce aflatoxins is reported to be highly variable; several strains are non-aflatoxinogenic because aflatoxin synthesis may become unstable in these fungi [6]. Moreover, the production of aflatoxins is regulated by various environmental conditions [7, 8] and nutrient sources [9]. Conventional methods using cultural media for aflatoxin production usually distinguish perfectly between aflatoxin-producing and non-producing strains [10]. Unfortunately, these methods are time-consuming and above all, as reported in the literature, they can fail to detect some aflatoxin-producing strains because the instability of aflatoxin production may occur in certain aflatoxinogenic strains growing in culture media [11, 12].

Further studies are needed to develop a rapid and more objective technique that permits clear differentiation between aflatoxin-producing and non-producing strains of *A. flavus* and *A. parasiticus*. Biosynthesis and the genetic background of aflatoxin production are well elucidated.

An overview about the genetic background of the aflatoxin biosynthetic pathway has been given by [13, 14]. Two different PCR systems for rapid detection of aflatoxinogenic fungi have been described [15, 16]. One is a multiplex system targeted against three genes of the aflatoxin biosynthetic pathway, in particular *nor-1*, the gene encoding for the synthesis of norsolonic acid, an intermediate in the aflatoxin biosynthetic pathway in *A. parasiticus*, which is converted to averantin (AVN) by a reductase/dehydrogenase enzyme; this reaction is reversible depending on NADPH or NADH [17]. The *ver-1* gene encoding for α-ketoreductase required for the conversion of versicolorin A (VERA) to demethylsterigmatocystin (DMST). The involvement of *omtA* in the later step of aflatoxin production has been established. This gene encodes *O*-methyltransferase A or *O*-methyltransferase II required for the conversion of ST (sterigmatocystin) to *O*-methylsterigmatocystin (OMST) and

[1] The article is published in the original.

[2] Corresponding author; e-mail: Moubasher54@hotmail.com

dihydrosterigmatocystin (DHST) to dihydro-*O*-methylsterigmatocystin (DHOMST) [18–20]. In the other system three genes have also been targeted (*nor-1*, *ver-1* and *aflR*); however, they are involved in single reactions [21, 22]. The *aflR* gene plays an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A*, *ver-1* and *nor-1* [23, 24]. The *aflR* gene encodes a sequence-specific DNA-binding binuclear zinc cluster (Zn(II) 2Cys6) protein, required for transcriptional activation of most, if not all, of the structural genes [22–24]. Ehrlich et al. (1998) suggested that the expression of *aflR* was regulated by environmental and nutritional factors that have long been known to affect aflatoxin formation [25].

Multiplex PCR with four sets of primers has permitted unequivocal detection of aflatoxinogenic strains of *A. flavus* and *A. parasiticus* by the presence of a complete pattern with four bands, thus indicating the presence of all the genes examined which encode for the functional products. The presence of a quadruplet pattern for some non-aflatoxinogenic strains of *A. flavus* indicates that the lack of aflatoxin production could also be due to simple mutations which lead to the formation of non-functional products.

The goal of the present work was to differentiate between aflatoxinogenic and non-aflatoxinogenic strains of *A. flavus* and *A. parasiticus* using conventional methods and a multiplex PCR procedure with four sets of primers.

MATERIALS AND METHODS

Eight strains of *A. flavus* and two strains of *A. parasiticus* used throughout this investigation, were isolated from different sources such as soil, contaminated fungal cultures, and contaminated tissue cultures. The isolates were identified according to [26]. Identification was confirmed by the Assiut University Mycological Centre (AUMC).

For the determination of aflatoxins production, three different media were used: Malt Glucose (MG), Czapek Dox (CD) and Yeast Extract Sucrose (YES). The spore suspension (100 μL) of each strain containing 10^7 spores/mL, prepared in 0.1% (vol/vol) Tween 20, was added to 100 mL of each tested medium in 250-mL Erlenmeyer flasks. After inoculation, the flasks were incubated on a rotary shaker at 26°C for 7 days. The mycelial mat was harvested by filtering through Whatman no. 1 filter paper. The filtrate was extracted twice with 100 mL chloroform in a separating funnel; the chloroform layer was filtered through anhydrous sodium sulfate. The chloroform extracts were combined and evaporated to dryness; hexane was added to remove fatty acids and nonpolar compounds from the residue, which was redissolved in 1 mL chloroform. Silica gel TLC plates (Sigma) were used for the aflatoxin analysis. Fifty microliters of each sample was spotted onto the TLC sheets. The TLC was developed in the toluene : ethyl acetate : acetic acid (50 : 30 : 4) solvent system. Pure aflatoxins (Sigma) were used as the standards. Aflatoxins were visualized under an UV lamp at 365 nm and their presence was confirmed chemically by spraying and with 50% H_2SO_4 and heating to charring.

DNA was extracted from 25 mg of the harvested mycelia, which was frozen in liquid N_2 and ground in a mortar, according to the protocol recommended for the DNA purification mini kit (Fermantas, GeneJet no. K0791). A typical PCR mixture contained: 3 μL DNA template, 6 μL *Tag* polymerase buffer, 1 × 25 μL primer (120 pmol mL^{-1} each), 25 μL H_2O and 1 μL *Tag* polymerase (1 U μL^{-1}). The initial denaturing cycle was at 95°C, 3 min; and then for 30 cycles 94°C 30 s; 60°C, 30 s; 72°C, 30 s; and the final extension at 72°C was for 10 min. The sequences of the primers used were as follows: nor1 (5'-ACCGCTACGCCG-GCACTCTCGGCAC-3'); nor2 (5'-GTTGGCCGC-CAGCTTCGACACTCCG-3') enclosing a 400-bp fragment (nucleotides 501–900) of the *A. parasiticus nor-1* gene; ver1 (5'-GCCGCAGGCCGCG-GAGAAAGTGGT-3'), ver2 (5'-GGGGATATA-CTCCCGCGACACAGCC-3'), enclosing a 537-bp fragment (nucleotides 623–1160) of the *A. parasiticus ver-1* gene; omt1 (5'-GTGGACGGACCTAGTC-CGACATCAC-3'), omt2 (5'-GTCGGCGCCACG-CACTGGGTTGGGG-3'), enclosing a 797-bp fragment (nucleotides 301–1098) of the *A. parasiticus omt-A* gene; aflR1 (5'-TATCTCCCCCC-GGGCATCTCCCGG-3'), aflR2, (5'-CC-GTCA-GACAGCCACTGGACACGG-3'), enclosing a 1032-bp fragment (nucleotides 450–1482) of the *A. parasiticus aflR* gene. All experiments were repeated three times.

RESULTS AND DISCUSSION

Identification of ten isolates was kindly confirmed by the Assiut University Mycological Centre, Assiut, Egypt (AUMC), where they were deposited in the culture collection. Six isolates (AUMC 9022, 9023, 9024, 9025, 9026 and 9031) were identified as *Aspergillus flavus* var. *columnaris* Raper & Fennell, while two isolates (AUMC 9027 and 9028) were identified as *Aspergillus flavus* Link, and two, as *Aspergillus parasiticus* Speare (AUMC 9029 and 9032) (Table 1). Aflatoxin production by the strains is shown in Table 2. Of ten strains, six (AUMC 9022, 9023, 9026, 9027, 9028 and 9031) were aflatoxin non-producers and only four (AUMC 9032, 9024, 9025 and 9029) were aflatoxin producers. *A. parasiticus* aflatoxin-producing strains released both aflatoxin B and G groups, while *A. flavus* strains produced mostly B rather than G group. *A. flavus* var. *columnaris* (AUMC 9022, 9023 and 9026) failed to produce both aflatoxin B_2 and G_2, while strain 9025 produced aflatoxins B_2 and G_2 on all three types of media. TLC analysis showed a clear differen-

Table 1. Identification of isolates by AUMS (Assiut University Mycological Centre)

AUMS no.	Identification
9022	*Aspergillus flavus* var. *columnaris* Raper & Fennell
9023	*Aspergillus flavus* var. *columnaris* Raper & Fennell
9024	*Aspergillus flavus* var. *columnaris* Raper & Fennell
9025	*Aspergillus flavus* var. *columnaris* Raper & Fennell
9026	*Aspergillus flavus* var. *columnaris* Raper & Fennell
9027	*Aspergillus flavus* Link
9028	*Aspergillus flavus* Link
9029	*Aspergillus flavus* Speare
9031	*Aspergillus flavus* var. *columnaris* Raper & Fennell
9032	*Aspergillus flavus* Speare

tiation between aflatoxin-producing and non-producing strains of *A. flavus* and *A. parasiticus*.

PCR was developed using four sets of primer for the *aflR*, *omt-A*, *ver-1*, and *nor-1* genes involved in the aflatoxin biosynthetic pathway. Figure and Table 3 show the results obtained for the strains examined. Bands of the fragments of the *aflR*, *omt-A*, *ver-1* and *nor-1* genes could be visualized at 1032, 797, 537 and 400 bp, respectively. Seven strains (AUMC 9032, 9022, 9023, 9024, 9025, 9029 and 9031) showed a quadruplet pattern, indicating the presence of the four genes of the aflatoxin biosynthetic pathway; two strains (AUMC 9026 and 9027) showed three genes, while AUMC 9028 showed only two genes.

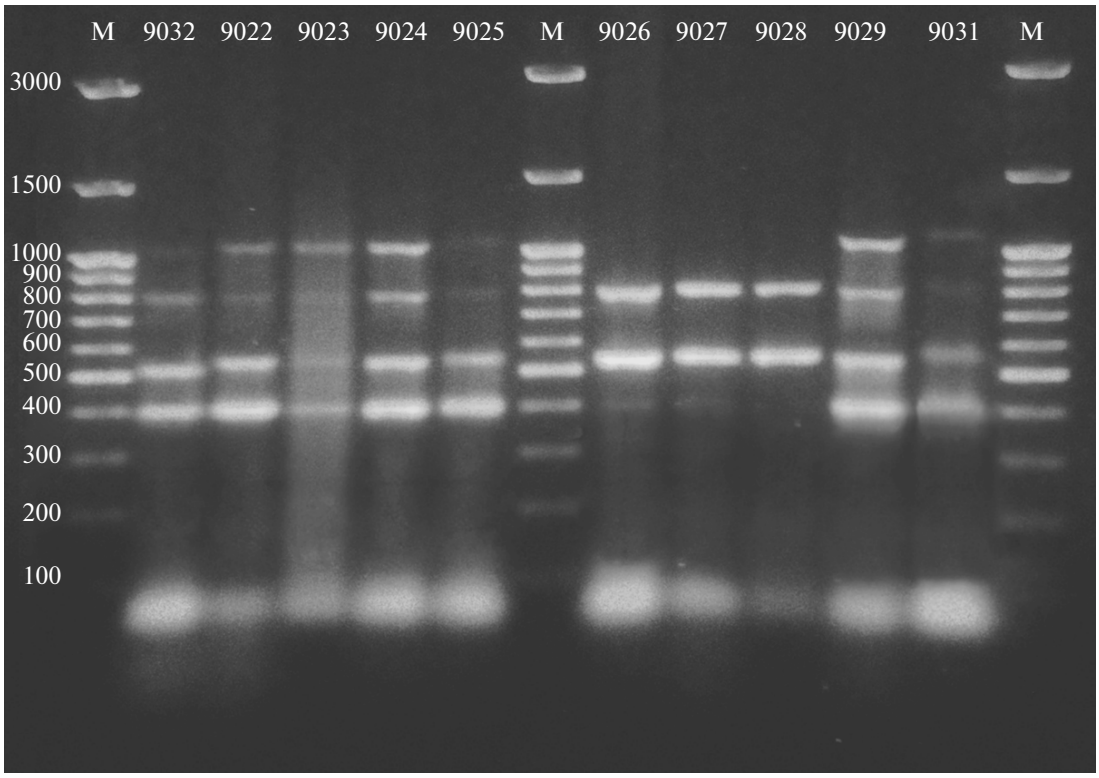
Table 3 compares the results obtained by PCR and by conventional methods. There was a correlation for aflatoxin-producing strains as regards aflatoxin production; a complete pattern with four bands obtained by PCR was always related to a positive response obtained by conventional methods. For the non-producing strains (AUMC 9022, 9023, 9031) there was no correlation between the results obtained by PCR and by conventional methods. The presence of a complete pattern in the non-aflatoxinogenic strains shows that it is not a sufficient marker for differentiation between aflatoxinogenic and some non-aflatoxinogenic strains. Lack of aflatoxin production apparently need not only be related to an incomplete pattern obtained in PCR. This suggests that different types of mutations may have inactivated the aflatoxin biosynthetic pathway of these strains [15].

In the present study an additional set of primers specific for the *aflR* gene was also used. This gene plays an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A*, *ver-1* and *nor-1* [22, 23]. Multiplex PCR with four sets of primers did not result in a clear detection of aflatoxinogenic strains of *A. flavus*, although it has permitted a clear detection of *A. parasiticus* by the presence of a complete pattern with four bands, (AUMC 9032 and 9029), thus indicating the presence of all the genes examined which encode the functional products. The presence of a quadruplet pattern for some non-aflatoxinogenic strains of *A. flavus* and *A. flavus* var. *columnaris* indicates that the lack of aflatoxin production could also be due to simple mutations (substitution of some bases) which lead to the formation of non-functional products. Aflatoxin production is controlled by a mechanism of regulation of structural gene transcription in which *aflR* plays a very important role. It was shown, by a time-consuming and very laborious hybridization technique [24] that AFLR, a product of the *aflR* gene, regulates the expression of the *omt-A* gene, a structural gene enclosed in the aflatoxin biosynthetic pathway. They found AFLR in all the non-aflatoxinogenic strains in *Aspergillus* section Flavi examined. However, *omt-A* was not expressed, even though it was present in all the strains.

Table 2. Production of aflatoxinus by the tested strains in different types of media

Strains	B_1			B_2			G_1			G_2		
	C	M	Y	C	M	Y	C	M	Y	C	M	Y
9032	–	+	+	–	+	+	–	+	+	+	+	+
9022	–	–	–/+	–	–	–/+	–	–	–/+	–	–	–/+
9023	–	–	–/+	–	–	–/+	–	–	–/+	–	–	–/+
9024	+	+	+	–	+	+	+	+	+	–	+	+
9025	+/–	+	+	–	+	+	–	+	+	+/–	+	+
9026	–	–	+/–	–	–	–/+	–	–	+/–	–	–	–/+
9027	–	–	+/–	–	–	–/+	–	–	+/–	–	–	+/–
9028	–	–	–	–	–	–	–	–	–	–	–	–
9029	–	+	+	–	+	+	+	+	+	+	+	+
9031	–	–	–/+	–	–	–/+	–	–	–/+	–	–	–/+

Czapek Dox, C; Malt Glucose, M; and Yeast Extract Sucrose, Y; very low –/+, low +/–.



Agarose gel electrophoresis of PCR products. Lane M is the marker ladder.

PCR has proved to be a very precise and rapid biomolecular technique for detecting aflatoxinogenic strains of *A. flavus*, *A. flavus* var. *columnaris*, and *A. parasiticus*, although it did not always permit discrimination between them and non-aflatoxinogenic strains. Indeed, the presence of the complete pattern with four bands in some non-aflatoxinogenic strains did not distinguish these strains from the aflatoxinogenic strains, in which a quadruplet pattern was always present. Conventional methods using cultural media for aflatoxin production distinguished perfectly between aflatoxin-producing and non-producing strains in the present study. Unfortunately, these methods are time-consuming and above all, as reported in the literature, they can fail to detect some aflatoxin-producing strains because instability of aflatoxin production may occur in certain toxigenic strains growing in culture media [11, 12].

Further studies are needed to develop a rapid and more objective technique that permits clear differentiation between aflatoxin- and non-aflatoxin-producing strains of *A. flavus* and *A. parasiticus*. RT-PCR was used for monitoring aflatoxin production in *A. parasiticus* [27]. The authors suggested that this assay had the potential of being able to detect a number of different mRNA transcripts from aflatoxin genes when the producing fungus was cultured under a variety of different physiological conditions affecting aflatoxin biosynthesis.

It would be interesting to use the RT-PCR biomolecular technique, analogous to that applied [28] for tricothecene-producing strains of *Fusarium* sp., as an important tool to differentiate aflatoxin-producing from non-producing strains of *A. flavus* and *A. parasiticus*. Indeed, RT-PCR enables the study of gene expression by allowing the detection of mRNAs transcribed by specific genes owing to PCR amplification of cDNA intermediates synthesized by reverse transcription. The presence or lack of mRNAs could per-

Table 3. Results obtained by four primes PCR and comparison to different media results

Strains	*afl R* 1032 bp	*omt-A* 800 bp	*ver-1* 537 bp	*nor-1* 400 bp	Aflatoxin production
9032	+	+	+	+	Positive
9022	+	+	+	+	Negative
9023	+	+	+	+	Negative
9024	+	+	+	+	Positive
9025	+	+	+	+	Positive
9026	–	+	+	+	Negative
9027	–	+	+	+	Negative
9028	–	+	+	–	Negative
9029	+	+	+	+	Positive
9031	+	+	+	+	Negative

mit direct differentiation between aflatoxinogenic and non-aflatoxinogenic strains. In addition, several specific mRNAs may be detected simultaneously in a single RNA sample by multiplex RT-PCR, with the advantage of having a unique response to the expression of several genes enclosed in the aflatoxin biosynthetic pathway.

REFERENCES

1. Cary, J.W., Ehrlich, K.C., Beltz, S.B., Harris-Coward, P., and Klich, M.A., Characterization of the *Aspergillus ochraceoroseus* aflatoxin/sterigmatocystin biosynthetic gene cluster, *Mycologia*, 2009, vol. 101, no. 3, pp. 352–362.
2. Henry, S.H., Bosch, F.X., and Bowers, J.C., Mycotoxins: risks in plant, animal and human systems, *Council Agric. Sci. Technol. Task Force*, 2003, report no. 139, p. 199.
3. Bhatnagar, D., Cary, J.W., Ehrlich, K., Yu, J., and Cleveland, T.E., Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development, *Mycopathol.*, 2006, vol. 162, pp. 155–166.
4. Pildain, M.B., Frisvad, J.C., Vaamonde, G., Cabral, D., Varga, J., and Samson, R.A., Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts, *Int. J. Sys. Evol. Microbiol.*, 2008, vol. 58, pp. 725–735.
5. Sepahvand, A., Shams-Ghahfarokhi, M., Jahanshiri, Z., Jamali, M., Allameh, A., and Razzaghi Abyaneh, M., A survey on distribution and toxigenicity of *Aspergillus flavus* from indoor and outdoor hospital environments, *Folia Microbiol.*, 2011, vol. 56, pp. 527–534.
6. Bennett, J.W. and Christensen, S.B., New perspectives on aflatoxin biosynthesis, *Adv. Appl. Microbiol.*, vol. 29, pp. 53–92.
7. Gqaleni, N., Smith, J.E., Lacey, J., and Gettinby, G., Effects of temperature and water activity, and incubation time on production of aflatoxins and cyclopiazonic acid by an isolate of *Aspergillus flavus* in surface agar culture, *Appl. Environ. Microbiol.*, 1997, vol. 63, pp. 1048–1053.
8. Keller, N.P., Nesbitt, C., Sar, B., Phillips, T.D., and Burrow, G.B., pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. *Phytopathol.*, 1997, vol. 87, pp. 643–648.
9. Moss, M.O., Economic importance of mycotoxins—recent incidence, *Int. Biodeterior.*, 1991, vol. 27, pp. 195–204.
10. Criseo, G., Bagnara, A., and Bisignano, G., Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group, *Lett. Appl. Microbiol.*, 2001, vol. 33, pp. 291–295.
11. Abarca, M.L., Bragulat, M.R., Bruguera, M.T., and Cabanes, F.J., Comparison of some screening methods for aflatoxigenic moulds, *Mycopathol.*, 1988, vol. 104, pp. 75–79.
12. Lemke, P.A., Davis, N.D., and Creech, G.W., Direct visual detection of aflatoxin synthesis by minicolonies of *Aspergillus* species, *Appl. Environ. Microbiol.*, 1989, vol. 55, pp. 1808–1810.
13. Woloshuk, C.P., Seip, E.R., Payne, G.A., and Adkins, C.R., Genetic transformation system for the aflatoxin-producing fungus *Aspergillus flavus, Appl. Environ. Microbiol.*, 1989, vol. 55, pp. 86–90.
14. Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H., and Leonard, T.J., Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans, Proc. Natl Acad. Sci. USA*, 1996, vol. 93, pp. 1418–1422.
15. Geisen, R., Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi, *Syst. Appl. Microbiol.*, 1996, vol. 19, pp. 388–392.
16. Shapira, R., Paster, N., Eyal, O., Menasherov, M., Mett, A., and Salomon, R., Detection of aflatoxigenic molds in grains by PCR, *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 3270–3273.
17. Bennett, J.W., Chang, P.K., and Bhatnagar, D., One gene to whole pathway: the role of norsolorinic acid in aflatoxin research, *Adv. Appl. Microbiol.*, 1997, vol. 45, pp 1–15.
18. Bhatnagar, D., Ullah, A.H.J., and Cleveland, T.E., Purification and characterization of a methyltransferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway, *Prep. Biochem.*, 1988, vol. 18, pp. 321–349.
19. Yabe, K., Ando, Y., Hashimoto, J., and Hamasaki, T., Two distinct *O*-methyltransferases in aflatoxin biosynthesis, *Appl. Environ, Microbiol.*, 1989, vol. 55, pp. 2172–2177.
20. Keller, N.P., Dischinger, J.H.C., Bhatnagar, D., Cleveland, T.E., and Ullah, A.H.J., Purification of a 40-kilodalton methyltransferase active in the aflatoxin biosynthetic pathway, *Appl. Environ. Microbiol.*, 1993, vol. 59, pp. 479–484.
21. Payne, G.A., Nystrom, G.J., Bhatnagar, D., Cleveland, T.E., and Woloshuk, C.P., Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus, Appl. Environ. Microbiol.*, 1993, vol. 59, pp. 156–162.
22. Chang, P.K., Yu, J., Bhatnagar, D., and Cleveland, T.E., The carboxy-terminal portion of the aflatoxin pathway regulatory protein AFLR of *Aspergillus parasiticus* Activates GAL1: lacZ gene expression in *Saccharomyces cerevisiae, Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 2508–2512.
23. Woloshuk, C.P., Foutz, K.R., Brewer, J.J.F., Bhatnagar, D., Cleveland, T.E., and Payne, G.A., Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 2408–2414.
24. Chang, P.K., Yu, J., Bhatnagar, D., and Cleveland, T.E., Repressor-AflR interaction modulates aflatoxin biosynthesis in *Aspergillus parasiticus, Mycopathol.*, 1999, vol. 147, pp. 105–112.
25. Ehrlich, K.C., Montalbano, B.G., Bhatnagar, D., and Cleveland, T.E., Alteration of different domains in aflR affects aflatoxin pathway metabolism in *Aspergillus parasiticus* transformants, *Fungal Genet. Biol.*, 1998, vol. 23, pp. 279–287.
26. Raper, K.B. and Fennell, D.I., The Genus *Aspergillus*, Hungtington, R.E., Ed., New York: Krieger, 1973.
27. Sweeney, M.J., Pamies, P., and Dobson, A.D.W., The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus* 439, *Int. J. Food Microbiol.*, 2000, vol. 56, pp. 97–103.
28. Doohan, F.M., Weston, G., Rezanoor, H.N., Parry, D.W., and Nicholson, P., Development and use of a reverse transcription-PCR assay to study expression of *tri5* by *Fusarium* species in vitro and *in planta*, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 3850–3854.