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AFB1 Occurrence and Aflatoxin Biosynthesis Genes in Wheat Derivatives

Mohamed A. Mahmoud[1] and Abeer R.M. Abd-El-Aziz[2]

[1]Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.
[2]Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia.

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The incidence of AFB1 (B1) was determined in 72 wheat derivatives samples (Biscuits, bread, cornflakes, flour, macaroni and wheat bran. Positive sample for biscuits, bread and flour (16.7%), cornflakes (8.33%), macaroni (33.33%) and wheat bran (50%). Sixteen samples were contaminated with B1 ranging from 0.21 to 2.17 μg/kg. PCR was used for the detection of B1 contamination based on aflatoxin biosynthetic genes *aflR*, *aflS*, *aflD*, *aflM* and *aflP*. The interpretation of the results revealed that PCR is a rapid and sensitive method.

Key words: AFB1, Wheat derivatives, *aflR*, *aflS*, *aflD*, *aflM* and *aflP*.

Wheat flour is an ingredient used in many foods and is one of the most important foods in the world. Flour is the cleanest end product of the milling process and is generally regarded as a microbiologically safe product as it is a low-activity commodity. However, *Aspergillus flavus* contaminate flour may survive for extended periods (Gashgari *et al*., 2010). The human consumption of wheat bran as a direct source of dietary fiber has grown in recent years, mainly because it is a cheap product and has a high dietary fiber content. *A. flavus* can be contaminated wheat bran (Trombete *et al*., 2014). Aflatoxin and fungi contamination of cornflakes and wheat-based pastas (macaroni, noodles and spaghetti has been well studied and documented (Ezekiel and Sombie, 2014). Aflatoxins (AFs) are toxic secondary metabolites produced by species of *Aspergilli*, especially *Aspergillus flavus*, *A. parasiticus*. These fungi can grow on certain foods and feeds under favorable conditions of temperature and humidity and generate AFs before and=or during harvest, handling, shipment, and storage (Frisvad and Thrane, 2004). The four major naturally occurring AFs are known as aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). AFs have been shown to be potent carcinogens, mutagens, and teratogens (Kotsonis *et al*., 2001).

Genetic studies suggest that the genes involved in the aflatoxin biosynthetic pathway are clustered (Bhatnagar *et al*., 2006). In general, the aflatoxin gene cluster in *A. flavus* consists of 25 genes spanning approximately 70 kb (Chang et al., 2005).

Aflatoxin biosynthesis in *A. flavus* is strongly dependent on the activities of regulatory proteins and enzymes encoded by four genes named *aflR*, *nor-1*, *ver-1* and *omtA*. By using specific PCR-based methods, the aflatoxigenic *A. flavus* isolates always show the complete gene set, whereas non-aflatoxigenic isolates lacking one, two, three or four PCR products (Criseo *et al*., 2008). Recently, several multiplex polymerase chain reaction (PCR) systems for aflatoxin-producing fungi were developed, capable of detecting several

* To whom all correspondence should be addressed.
E-mail: aabdelaziz@ksu.edu.sa

genes coding for enzymes at different stages of the aflatoxin biosynthesis pathway (Criseo *et al*., 2001; Chen, *et al*., 2002).

The objectives of this study were to (a) AFB1 level in wheat derivatives samples collected from different region in Riyadh by HPLC (b) use of different biosynthetic and regulatory genes as indicators of aflatoxin contamination for wheat derivatives samples.

MATERIALS AND METHODS

Extraction of aflatoxin from wheat derivatives samples

We collected 72 wheat derivatives samples for extraction of AFB1 (B1), 20 g of each sample was mixed with 100 ml of 4% acetonitrile aqueous solution of potassium chloride (9:1). Extraction was followed by shaking for 20 min and filtered through Whatman No.4 filter paper under vacuum condition. For purification, 100 ml of n-hexane were added to the filtrate and shaken for 10 min. After separating, the upper phase (n hexane) was discarded. To the lower phase, 50 ml deionized water and 50 ml chloroform were added and this solution was shaken for 10 min. After separation, the lower phase was collected and the upper phase was re-extracted twice with 25 ml of chloroform by using the above conditions. Then the chloroform was evaporator in a 40°C water bath at low speed. Methanol at the rate of 2 ml was added and the solution filtered through a 0.45 µl filter (Zaboli *et al*, 2011).

PCR for aflatoxin biosynthesis genes

Genomic DNA was extracted according to (Amer *et al*., 2011). PCR reaction five pairs of primers were used on the basis of the sequences of aflatoxin biosynthetic genes *aflR, aflS, aflD, aflM* and *aflP*. The housekeeping gene tub1 coding for ²-tubulin (primer pair tub1-F/tub1-R) was used as internal amplification control (IAC). Sequences of primers are listed in Table 1. Amplification was performed in 25 µL reaction, containing reaction buffer (20 mM Tris- HCl, pH 8.4, 50 mM KCl), 3.4 mM MgCl2, 0.25 mM dNTPS, 0.4 mM of each primer, 2 U Taq DNA polymerase (BioLabs), and 25 ng genomic DNA. Amplification parameters (*aflR, aflS, aflD, aflM* and *aflP*) consisted of 1 cycle 95 °C (1 min), 60 °C, (30 s), 72 °C (30 s) and 34 cycles at 94°C (30 s), annealing at 65°C (30 s), and extension at 72°C (6 min). PCR products were separated by 1.5% agarose gel, stained with ethidium bromide in 1X TAE buffer (Tris-acetate EDTA, pH 8.0) at 100 V for 50 min, using a 100-bp ladder DNA marker (Intron, Korea). The DNA gel was scanned for band Rf using a gel documentation system (AAB Advanced American Biotechnology, Fullerton, CA, USA).

RESULTS

Survey of seventy two wheat derivatives

Survey of seventy two wheat derivatives, marketed in Riyadh, Saudi Arabia, was analyzed for the presence of aflatoxins B1 (Table 2). High level of B1 2.17 (wheat bran) µg/kg and low level of

Table 1. Primers used in this study, target gene, sequence and expected PCR product size

Primer code	Target gene	Primer sequences	PCR product size (bp)	References
nor-1	*aflD*	5' -ACCGCTACGCCGGCACTCTCGGCAC-3'	400	Criseo *et al*. (2008)
nor-2		5' -GTTGGCCGCCAGCTTCGACACTCCG-3'		
ver-1	*aflM*	5' -GCCGCAGGCCGCGGAGAAAGTGGT-3'	737	
ver-2		5' -GGGGATATACTCCCGCGACACAGCC-3'		
omt-1	*aflP*	5' -GTGGACGGACCTAGTCCGACATCAC-3'	799	
omt-2		5' -GTCGGCGCCACGCACTGGGTTGGGG-3'		
aflR-1	*aflR*	5' -TATCTCCCCCCGGGCATCTCCCGG-3'	1032	
aflR-2		5' -CCGTCAGACAGCCACTGGACACGG-3'		
AflJ-gF	*aflS*	5' -GAACGCTGATTGCCAATGCC-3'	1399	Degola *et al* (2007)
AflJ-giR		5' -CGGTCAGGATGTTACTAAGC-3'		
Tub1-F	tub1	GCT TTC TGG CAA ACC ATC TC	1406	Scherm *et al*. (2005)
Tub1-R		GGT CGT TCA TGT TGC TCT CA		

Table 2. AFB1 level in wheat derivatives samples collected from different region in Riyadh

Sample no.	Location	Biscuits	Bread	Cornflakes	Flour	Macaroni	Wheat Bran
Simple1	North	nd	0.28	nd	nd	0.53	nd
Simple2		nd	nd	nd	nd	nd	2.17
Simple3		nd	nd	nd	nd	nd	nd
Simple4	South	0.21	0.46	nd	nd	nd	1.29
Simple5		nd	nd	nd	nd	nd	nd
Simple6		nd	nd	0.59	nd	nd	0.82
Simple7	West	0.33	nd	nd	1.37	0.68	1.15
Simple8		nd	nd	nd	nd	nd	nd
Simple9		nd	nd	nd	nd	nd	0.94
Simple10	East	nd	nd	nd	nd	0.35	nd
Simple11		nd	nd	nd	nd	nd	0.76
Simple12		nd	nd	nd	0.78	0.29	nd

Nd = not detected

Table 3. Low and high levels of AFB1 wheat derivatives samples collected from different region in Riyadh

Wheat derivatives	n	Positive sample	low level of B1 (µg/kg)	High level of B1 (µg/kg)
biscuits	12	16.67	0.21	0.33
bread	12	16.67	0.28	0.46
cornflakes	12	08.33	0.59	0.59
flour	12	16.67	0.78	1.37
macaroni	12	33.33	0.29	0.68
Wheat bran	12	50	0.76	2.17

Table 4. Presence of aflatoxin genes in wheat derivatives samples collected from different region in Riyadh

Sample name	AFB1	β-tubulin	Aflatoxin genes				
			aflR	*aflS*	*aflD*	*aflM*	*aflP*
Biscuits 1	+	+	+	+	+	+	+
Biscuits 2	+	+	+	+	+	+	+
Bread 1	+	+	+	+	+	+	+
Bread 2	+	+	+	+	+	+	+
Cornflakes 1	+	+	+	+	+	+	+
Flour 1	+	+	+	+	+	+	+
Flour 2	+	+	+	+	+	+	+
Macaroni 1	+	+	+	+	+	+	+
Macaroni 2	+	+	+	+	+	+	+
Macaroni 3	+	+	+	+	+	+	+
Macaroni 4	+	+	+	+	+	+	+
Wheat bran 1	+	+	+	+	+	+	+
Wheat bran 2	+	+	+	+	+	+	+
Wheat bran 3	+	+	+	+	+	+	+
Wheat bran 4	+	+	+	+	+	+	+
Wheat bran 5	+	+	+	+	+	+	+
Wheat bran 6	+	+	+	+	+	+	+
controls							
Biscuits	-	+	+	-	+	-	+
Bread	-	+	-	+	+	-	-
Cornflakes	-	+	+	+	-	+	-
Flour	-	+	-	+	-	+	+
Macaroni	-	+	+	-	+	+	-
Wheat bran	-	+	+	+	-	-	+

B1 0.21 µg/kg (Biscuits) (Table 2). Biscuits bread and flour (16.7%), cornflakes (8.33%), macaroni (33.33%) and wheat bran 50% table 2.

Amplification patterns of aflatoxin biosynthesis genes (Table 3)

The presence or absence of the aflatoxin biosynthetic genes in seventeen samples (all positive samples) wheat derivatives (Table 4). Seventeen samples showed DNA fragments that correspond to the complete set of genes comparing with controls.

DISCUSSION

The mycological profile of the retail wheat flour selling in different markets at Jeddah (Kingdom of Saudi Arabia) was studied. The most common genera were *Aspergillus* (isolated from 70% of the tested samples), *Penicillium* (30%), *Eurotium* (14%), and in a lesser extent *Fusarium* (20%) and *Alternaria* (18%) (Gashgari *et al*., 2010). One hundred and eight samples of whole-wheat grains (n=35), wheat bran (n=32), whole-wheat flour (n=26) and refined wheat flour (n=15) marketed in hypermarkets, supermarkets and health food stores were analyzed by High Performance Liquid Chromatography with fluorescence detection (HPLC-FL). Thirty-three samples (30.6%) were positive for at least one aflatoxin and the B1 form had the highest prevalence in the samples (Trombete *et al*., 214). *Aspergillus* was the genus most detected at high frequency in all of the wheat flour samples from Algeria (Riba *et al*., 2008). Roige *et al*. (2009) reported that *Penicillium* (42%), *Fusarium* (27%), and *Alternaria* (25%) were the most frequently recovered genera from wheat in Argentina.

PCR showed that all strains contained the four aflatoxin genes examined, regardless of expression profiles. Our results also showed that *aflD* expression is a reliable marker to aflatoxin contamination of wheat derivatives samples (Abdel-Hadi *et al*., 2011). Using PCR we showed that all aflatoxin and non-aflatoxin producers harbour the four genes (*aflD*, *aflM*, *aflP* and *aflR*) examined. This discounts the possibility that a lack of aflatoxin production in certain strains is due to loss of the genes from the genome. Chang *et al*. (2005) who reported that the loss of production of aflatoxins B1 and B2 in many 10 nonaflatoxigenic *A. flavus* isolates is not caused by large deletions or a complete loss of the aflatoxin gene cluster, but probably results from point mutations. PCR was used for the detection of aflatoxinogenic *Aspergilli* based on *nor-1*, *ver-1*, *omt-1* and *aflR*. The interpretation of the results revealed that PCR is a rapid and sensitive method (Erami *et al*., 21007).

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