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التغيرات المستحدثة بسموم الأفلاتوكسين ب1 للكروموسومات والجينات الريبوسومية في نبات القمح

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الملخص:

يعد الأفلاتوكسين ب1 أحد النواتج الأيضية الثانوية التي تفرزها بعض الفطريات وهو بدوره يسبب العديد من الأمراض الخطيرة للنبات والحيوان والإنسان. ولقد أكدت الدراسات السيتولوجية والجزيئية التي أجريت في هذا البحث على نبات القمح السمية الجينية للأفلاتوكسين ب 1 تحت التركيزات المختبرة وهي (5 ، 10 ، 15 ، 20 ، 25 ميكروجرام/مل). كما أشارت الدراسات السيتولوجية إلى حدوث أنواع مختلفة من التشوهات في كروموسومات القمح أثناء الانقسام الميوزى؛ فقد تم رصد خلايا تحتوي على كروموسومات متلزنة وأزواج كروموسومات شاردة وجسور كروموسومية وكروموسومات متلكئة وانقسام نووى غير متماثل، وأيضا وجود نويات صغيرة في خلايا الطور البينى. وقد لوحظ أن النسبة المئوية الكلية للتشوهات الكروموسومية تزداد بالزيادة التدريجية في تركيز الأفلاتوكسين ب 1 حتى تصل لأعلى قيمة (2.9%) عند تركيز 15 ميكروجرام/مل ثم تنخفض مرة أخرى إلى (1.7%) عند أعلى تركيز للأفلاتوكسين ب 1 وهو 25 ميكروجرام/مل. وقد أظهرت الدراسات الجزيئية بعض التغيرات على أنماط الفصل الكهربى لحزم الدنا DNA الناتجة من تفاعل البلمرة المتسلسل (PCR) باستخدام الباد ئ الخاص بتتابع دنا الريبوسومى (5S rDNA)، وبينما كان حجم هذه الحزم 100 ،400 ،500 ،800 ،900 زوج من القواعد النيتروجينية في نباتات الكنترول، فقد اختفت بعض هذه الحزم في النباتات المعالجة بالأفلاتوكسين ب1، وهذا يشير إلى احتمال حدوث تغيرات يتم توارثها في تتابع الدنا المسئول عن تكوين الحمض النووى الريبوزى الريبوسومى rRNA.

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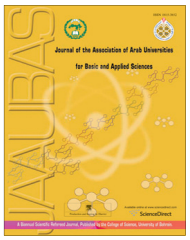

ORIGINAL ARTICLE

Aflatoxin B_1 induces chromosomal aberrations and 5S rDNA alterations in durum wheat

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Abstract Aflatoxin B_1 is a secondary metabolite of some fungi that causes very serious diseases in plants, animals and humans. Both cytological studies and molecular techniques revealed that AFB_1 at tested concentrations of 5, 10, 15, 20 and 25 μg/ml exhibits genotoxic effect in wheat plants. Several types of chromosomal aberrations have been detected during meiosis; these aberrations include chromosome stickiness, outside bivalents, bridges, laggards, unequal division and micronuclei. The percentage of total abnormalities increased gradually with the increase of toxin concentration but declined again at the highest concentrations. The highest value (2.9%) of abnormalities, detected during meiosis, was in plants treated with 15 μg/ml AFB_1 even so, this value decreased to 1.7% in plants treated with the highest concentration (25 μg/ml) of AFB_1. The 5S primer generated amplified DNA fragments of 100, 400, 500, 800 and 900 bp in control plants. However, some of these fragments were missing or faint in a number of AFB_1-treated plants. Such considerable alterations in DNA profiles of 5S primed amplicons might indicate the generation of various alterations in the inherent property of the 5S rRNA gene sequence.

1. Introduction

Mycotoxins are secondary metabolic products from moulds which can grow on the plant either in the field or during storage (El-Naghy et al., 1991) and are potentially toxic for human beings, animals and plants (Fadl-Allah, 1987 and Helmey, 2003). These toxins are found as natural contaminants in many foodstuffs of plant origin, particularly cereals but also fruits, hazelnuts, almonds, seeds, fodder and foods consisting of or manufactured from these products and intended for human or animal consumption. Mycotoxins can be simply classified according to their major toxic effects. Amongst the groups of mycotoxins, which have been considered being important from a processed-foods and health perspective are the aflatoxins (French Food Safety Agency, Summary report, 2006). Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus* and *A. parasiticus*; in particular, *A. flavus* which is a common contaminant in agriculture. Aflatoxin B_1 is the most potent natural carcinogen known (Squire, 1981) and is usually the major aflatoxin produced by toxigenic strains. The mutagenicity of aflatoxin B1 to animals and humans is believed to

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involve oxidative activation by a cytochrome P_{450}, in the liver and the kidney. An epoxide is created at the 8, 9 position in AFB_1 that reacts with the N^7-guanine residues of DNA, causing mutations in the tumor repressor gene p53 (Minto and Townsend, 1997).

The structure, organization, and evolution of the 5S rRNA (ribosomal ribose nucleic acid) multi-gene family have been studied in detail in the Triticeae (Dvorak et al., 1989 and Scoles et al., 1988). The 5S rRNA multigenes exist in two sizes: the long unit (ca. 500 bp), consisting of a 120-bp coding and 380-bp spacer region, and a short unit (ca. 400 bp), consisting of a coding region of the same length (120-bp) and a smaller spacer nine region (ca. 280 bp). Plant 5SrDNA is organized in tandemly repeated arrays that occur at one or more chromosome loci (Goldsbrough et al., 1981; and Sastri et al., 1992). The 5S rDNA repeat usually consists of a 120-base pair genic region and a nontranscribed spacer of variable length. The 120-bp genic region is conservative and can be aligned well at broad taxonomic levels (Szymanski et al., 1998). The intergenic spacer region is much more variable among plant taxa and ranges in size from 100 to 700 bp (Cox et al., 1992; and Sastri et al., 1992). The number of repeats per genome can vary from less than 1000 to over 100000 (Schneeberger et al., 1989; Sastri et al., 1992; and Cronn et al., 1996).

The objective of the present study was to determine the involvement of other possible mechanisms in Aflatoxin B_1 (AFB_1) induces toxicity. The applied material was the durum wheat plant (*Triticum durum*) which considered as one of the most important crop plants in Egypt.

2. Materials and methods

Grains of Durum wheat variety Beni-sueif 1) that has been used in this study of this wheat were kindly obtained from Seds Research Center Beni-sueif governorate, Egypt. The pure *Aspergillus* toxin Aflatoxin B_1 (Fig. 1) used in the present study, was obtained from Sigma chemical company, USA. Five different concentrations (5, 10, 15, 20, 25 μg/ml) of this toxin were employed. Aflatoxin B_1 was dissolved in 70% ethyl alcohol and the appropriate dilutions were prepared with sterile distilled water.

2.1. Grain germination

Similar mature grains of durum wheat were selected and surface sterilized using 70% ethanol followed by H_2O_2 for 3 min., rinsed in sterile distilled H_2O three times and allowed to dry on sterile filter paper. Grains were then soaked in sterile H_2O for 24 h and then soaked in the appropriate concentrations of the toxin. As a control, grains were soaked in sterile ddH_2O free of toxin, for the same time. Grains were grown in the field in December 2005 season. Each line was planted as 30 plants/row. Numbers of tillers of 10 randomly chosen plants were scored at flowering stage.

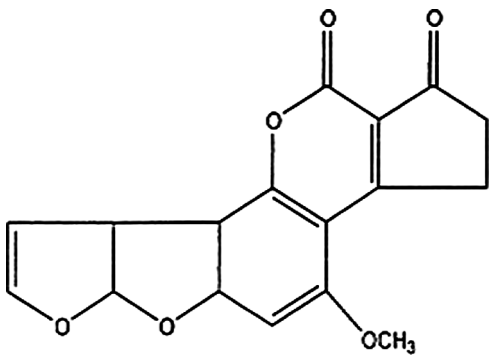

Figure 1 Formula of aflatoxin B_1 (AFB_1).

2.2. Cytological methods

Cytological effects of Aflatoxin B_1 were studied by performing meiotic chromosome analysis at first and second stages of meiotic division. Young flowering buds of wheat were collected at 8–9 o'clock in the morning and immediately fixed in a freshly prepared mixture of ethyl alcohol and glacial acetic acid (3:1 v/v) for 24 h. Pollen mother cells (PMCs) were prepared excised from the fixed anthers and stained with aceto-carmine (1%). Chromosomal aberrations in treated plants compared to the control were scored in at least 3–5 slides of each concentration.

2.3. Molecular studies

2.3.1. DNA extraction

Fresh young leaves of the previously selected 10 plants of each concentration were collected. About 100–300 mg of leaves of each sample were grinded in liquid nitrogen to a fine powder, transferred to 1.5 ml Ependorf tube then 500 μl of Cornel extraction buffer (500 mM NaCl; 100 mM Tris–HCl, pH 8.0; 50 mM EDTA and 0.84% SDS) pre-warmed to 65° C was added. The tubes were placed at 65 °C in water bath for 45 min. After cooling slightly to the room temperature, the specimens were washed by using the same volume (~500 μl) of phenol; phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1), respectively at room temperature. About 400 μl supernatant were drawn off into a new 1.5 ml tube containing 1 ml of 100% cold ethanol. DNA was precipitated by centrifugation at 13000 rpm for 10 min then washed with 1 ml 70% cold ethanol. After drying, pellet of DNA was dissolved in 50 μl of re-hydration buffer, and then stored at 4 °C DNA was verified by 1% agarose gel electrophoresis and its concentration and purity were determined with a spectrophotometer at 260 and 280 nm absorbance as mentioned by Sambrook et al. (1989).

2.3.2. PCR conditions (5S primer)

Two specific 5S oligo-nucleotide primers (forward and reverse) were used for screening the 5S rDNA region(s) in the mitotic chromosomes of Durum wheat. The primers were 20-base long with the following sequence:

5′- CGGTGCATTAATGCTGGTAT-3′ forward

5′- CCATCAGAACTCCGCAGTTA- 3′ reverse

DNA amplifications were performed in a final volume of 50 μl containing 25 μl 2× master mix (0.05units/μl Taq DNA polymerase in 2× PCR buffer [4 mM $MgCl_2$ and 4dNTPs (0.4 mM of each)], 10 μM of primer and (1 ng) of DNA template. The final reaction volume was completed to 50 μl using sterilized double distilled water.

The amplifications were carried out in a thermal cycler (Thermo Hybaid, Franklin, USA) programmed for initial preheating period in one step of 5 min. at 94 °C; subsequent 30 cycles of three steps in each, the first step was DNA denaturation

Table 1 The mean number of tillers/plant treated with different concentrations of AFB_1.

Concentrations	Mean number of tillers
Control	5.3
5 μg/ml AFB_1	2.3
10 μg/ml AFB_1	4.6
15 μg/ml AFB_1	4.9
20 μg/ml AFB_1	4.4
25 μg/ml AFB_1	3.7

at 94 °C for 45 s, followed by the step of primer annealing at 56 °C for 45 s and the third step of primer extension at 72 °C for 45 s; subsequent the final cycle in one step of post extension at 72 °C for 10 min. Amplification products were resolved by gel electrophoresis on 1.5% agarose gels in Tris–acetate EDTA (TAE) buffer for 1 h at 80 V. Subsequently, gels were stained with ethidium bromide (0.1 g ethidium bromide dissolved in 10 ml 1× TAE buffer) for 30 min., visualized on UV light and photo-documentation was performed. DNA fragments sizes were estimated by comparison with the standard marker of 1 Kb ladder.

3. Results and discussion

3.1. Germination

The Number of tillers in 10 randomly chosen plants was scored after three months of planting in the field (Table 1). The highest number of tillers was found in control plants whereas, plants treated with 5 μg/ml AFB_1 showed the lowest numbers of tillers. However, the numbers of tillers were increased gradually by increasing toxin concentration up to 15 μg/ml conversely; tillers number was declined again at the highest concentrations. The highest value (4.9) was in plants treated with 15 μg/ml AFB_1 even so, this value decreased to 3.7 in plants treated with the highest concentration (25 μg/ml) of AFB_1. Similar effects of Aflatoxin were reported by Crisan (1973) in *Lepidium sativum*. He found that, concentrations more than 10 μg/ml of aflatoxin, induced the maximal reduction in the rate of growth of hypocotyls after germination. His results were discussed in relation to the effects of Aflatoxin on DNA dependent RNA biosynthesis.

Reduction in number of tillers in plants treated with high concentrations of Aflatoxin may be due to the accumulation of DNA damage in cells, which leads to apoptosis. This may be similar to the effect of Zeralenone, which arrest cell cycle and induces apoptosis in cultured DOK cells as reported by Salwa et al. (2003). She reported that, the apoptotic pathway is the only option for a cell when DNA repair systems are overburdened due to too many damages. Vogelstein and Kinzler, 1992 have shown that among its diverse functions, the p53 gene normally prevents DNA replication in cells that have DNA damage by maintaining the cell in G2/M phase allowing more opportunity for DNA repair. Cells with inactivated p53 might therefore survive abnormally and allow further DNA damage to accumulate (Lane, 1992), a situation, which favours carcinogenesis (Symonds et al., 1994).

Table 2 The frequency and types of chromosomal abnormalities induced during first meiosis in the PMSc of plants treated with different concentrations of Aflatoxin B_1.

AFB_1 Concentration (μg /ml)	Meiosis I															
	Diakinasis			Metaphase I			Anaphase I				Telophase I			Total		
	Normal	Sticky	Out-side	Normal	Sticky	Out-side	Normal	Lag	Bridge	Fragment	Normal	Lag	Fragment	Normal	Abnormal	Aberrations%
Control	252	1	1	150	4	1	52	2	3	1	145	-	-	599	13	2.12
5	114	3	4	310	2	-	68	-	13	-	333	-	-	825	22	2.6
10	142	-	-	188	9	13	103	-	9	-	443	2	-	876	33	3.63
15	240	5	7	145	1	3	102		11		168	3	-	655	30	4.38
20	131	7	2	369	15	20	127	1	17	-	498	-	4	1125	66	5.54
25	165	7	1	235	9	25	33	-	6	-	125	-	-	558	48	7.92

Table 3 The frequency and types of chromosomal abnormalities induced during the second meiosis in the PMCs of plants treated with different concentrations of Aflatoxin B_1.

AFB_1 Concentration (μg/ml)	Meiosis II												
	Metaphase II			Anaphase II				Telophase II		Tetrad		Total		
	Normal	Sticky	Out-side	Normal	Lag	Bridge	Unequal-division	Normal	Micro-nuclei	Normal	Micro-nuclei	Normal	Abnormal	Aberration %
Control	185	1	2	151	–	4	4	207	4	–	–	543	15	2.69
5	36	2	–	38	–	1	–	122	1	80	2	276	6	2.13
10	104	18	–	102	1	4	6	137	1	210	–	553	30	5.14
15	95	21	–	8	1	1	1	285	1	127	2	515	27	4.98
20	210	5	–	72	1	–	3	108	–	–	–	390	9	2.25
25	85	3	8	57	–	3	6	133	1	182	4	457	25	5.18

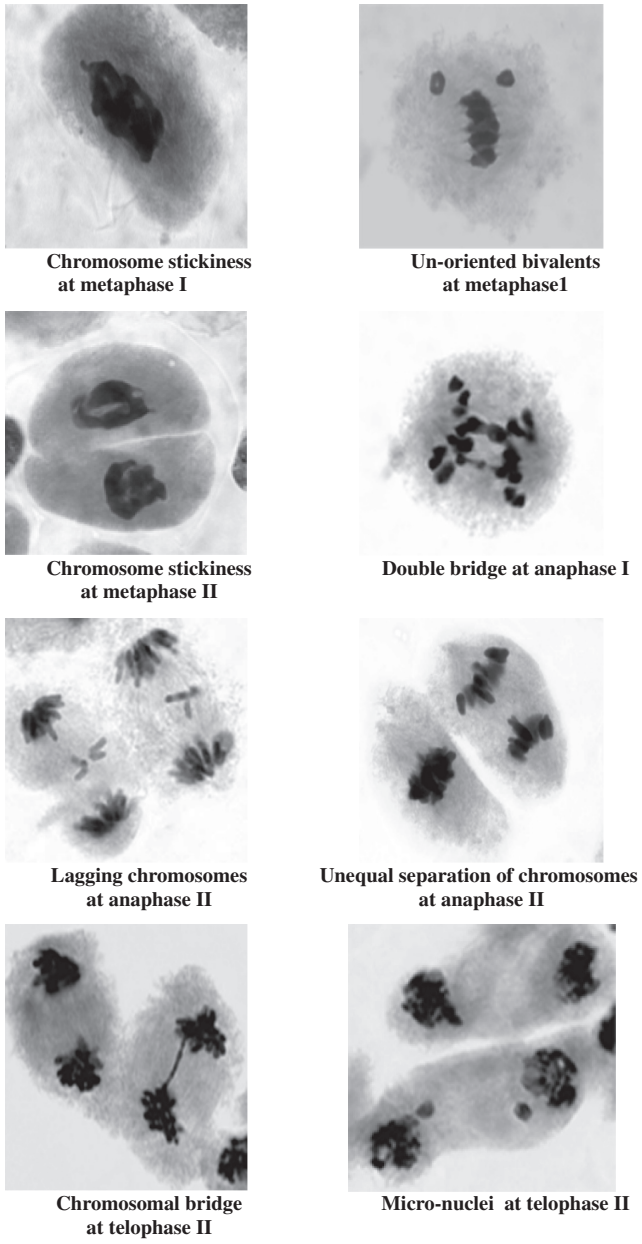

Figure 2 Types of chromosomal aberrations induced by AFB_1 in the PMCs of wheat plants during 1st meiotic and 2nd divisions.

3.2. Cytogenetic effects

The types and frequency of chromosomal aberrations during first and second meiotic division of wheat plants treated with different concentrations of Aflatoxin B_1 were analyzed. At least three slides of each concentration were examined. The common types of chromosomal irregularities, which have been recorded in the present study were stickiness, laggards, bridges, fragments, unequal division (lag-division) and micro-nuclei. The meiotic values of normal cells and types of chromosomal irregularities induced by AFB_1 during the first and second meiotic divisions are shown in Tables 2 and 3, respectively. The results showed that, Aflatoxin B_1 provoked several chromosomal irregularities in wheat at different stages of meiotic division. (Tables 2 and 3).

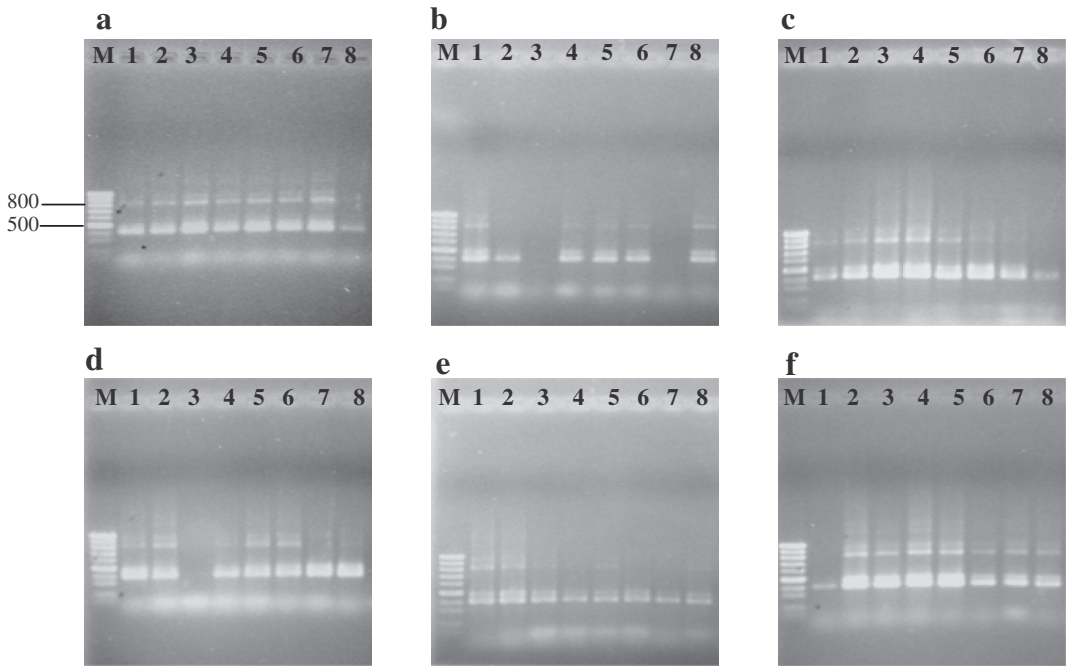

Figure 3 DNA amplification patterns showing the effect of different concentrations of Aflatoxin B_1 on eight wheat plants using the specific 5S DNA primer. (a) Control (b) 5 AFB_1 (c) 10 AFB_1 (d) 15 AFB_1 (e) 20 AFB_1 (f) 25 AFB_1.

Regarding the first meiotic division, data in Table 2 showed that out of 599 PMCs of the control plants, 13 were abnormal with a percentage of 2.12%. Plants treated with 5 μg/ml AFB_1 revealed 825 normal PMCs and 22 abnormal cells with 2.6% abnormalities. The percentage of abnormalities was increased up to 3.63 in plants treated with 10 μg/ml of AFB_1. Plants treated with 15 μg/ml AFB_1showed 4.38% abnormalities. The percentage of abnormalities was further increased up to 5.54% upon treating with 20 μg/ml of $AFB_1$and reached to the highest value of 7.92 in plants treated with 25 μg/ml AFB_1.

In the second meiotic division, control plants exhibit 543 normal PMCs and 15 abnormal cells with a percentage of 2.69% (Table 3). Relating to the control, the lowest concentration (5 μg/ml) of AFB_1did not reveal any effect on the percentage of abnormalities, as it is provoked six abnormal cells out of 276 PMCs in the second meiotic division with a percentage of 2.13%. The percentage of abnormalities was increased to 5.14% in plants treated with 10 μg /ml AFB_1. Plants treated with 15 μg /ml AFB_1showed 4.98% of abnormal cells. Data also revealed that plants treated with 20 μg/ml AFB_1 showed 2.25% abnormal cells. Whereas, the percentage of abnormal cells detected in plants treated with the highest concentration used in this study (25 μg/ml) of AFB_1 was 5.18%. In brief the percentage of chromosomal abnormalities induced by AFB_1 in the first and second meiotic divisions are clearly dose dependant, as it increased as the concentration of the toxin increased and the duration of the treatment prolonged.

Examples of chromosomal abnormalities produced in the PMCs of plants treated with different concentrations of toxin during the 1st and the 2nd meiosis are shown in Fig. 2. Bridges at anaphase could be explained according to the principle that, broken chromosome ends exhibit a tendency to fuse and form dicentric chromosomes (Werner et al., 1992). These dicentrics usually give rise to chromatin bridge-fusion-bridges at anaphase and may break at telophase, thus perpetuating the break (BFB) cycle after fusion of the newly broken chromosome ends.

3.3. Molecular studies

3.3.1. The 5S rDNA specific primer

In the present investigation the specific 5S DNA primer has been used to detect the possible mutagenic effects of the toxins at the genome level in wheat seedlings. The PCR reactions using this primer showed that DNA amplicons posses some variations in the DNA profiles within the examined samples as compared with the control pattern. Such variations include band intensity and appearance of novel bands or disappearance of others. DNA amplification patterns of wheat plants treated with AFB_1 have been shown in Fig. 3.

The number and the intensity of the amplified DNA fragments produced by PCR using 5S primer are listed in Table 4. Plants treated with aflatoxin B_1 showed some changes in the 5S primed amplification profiles as compared with to the control ones. Data in table 4 show that 5S DNA primer generated a band of 100-bp, which is clear in all plants except plant numbered three, seven, eight treated with 5 μg/ml AFB_1, which posses faint bands. However, the amplified fragment of 400-bp was present in all control and treated plant, while it was absent in plant numbered three, seven treated with 5 μg/ml AFB_1 and plant numbered three treated with 15 μg/ml AFB_1.

The 5S DNA primer also displayed a 500-bp band, which was clear in almost all plants while it was faint in the plant No. 2 treated with 5 μg/ml AFB_1 and plant No. 1 treated with 10 μg/ml AFB_1, that was absent in the plant No. 8 of the control, plants numbered three and seven treated with 5 μg/ml AFB_1, plant No. 8 treated with 10 μg/ml AFB_1, plant No. 3 treated with 15 μg/ml AFB_1 and plant No. 1 treated with 25 μg/ml AFB_1. Data also show that, the amplified DNA

Table 4 The presence and absence of amplified DNA fragments generated by 5S DNA primer in wheat plants treated with AFB_1.

Base No.	MW (bp)	Control				5				10														
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
5	900	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	*	*	*	*	–	–	–
4	800	+	+	+	+	+	+	+	–	+	–	–	–	*	*	–	+	*	+	+	+	+	+	–	–
3	500	+	+	+	+	+	+	+	–	+	*	–	+	+	+	–	+	*	+	+	+	+	+	+	–
2	400	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+
1	100	+	+	+	+	+	+	+	+	+	+	*	+	+	+	*	*	+	+	+	+	+	+	+	+
Base No.	MW (bp)		15							20								25							
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
5	900	–	*	–	–	*	*	–	–	+	+	–	–	–	–	–	–	–	+	–	+	+	–	–	–
4	800	*	+	–	*	+	+	–	–	+	+	–	–	+	–	–	+	–	+	+	+	+	+	+	+
3	500	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
2	400	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) Present and clear (*) present and faint (–) absent.

fragments of 800-bp and 900-bp were amplified in few plants but was missing in many others compared to the control profile.

The presence or absence of a particular DNA band depends on the presence or absence of the DNA sequence to be amplified within the genome (Michelmore et al., 1991 and Abdel-Razik, 1998). Based on such assumption, it could be suggested that treatment of wheat plants with toxins might induce DNA rearrangements or deletions that lead to the observed changes. Yang and Quiros (1993) reported that, the intensity of DNA band depends on the starting copy number of a particular DNA sequence within the genome. Therefore the change in band intensity could be interpreted on the basis of deletion or duplication of some DNA sequences.

Induction of chromosomal aberrations as well as changes in amplified DNA profiles may reflect the direct effect of Aflatoxin B_1 on DNA molecule. In animals, the mutagenicity of aflatoxin B_1 is believed to involve oxidative activation by a cytochrome P450 in the liver and the kidney. Cytochrome P450 enzymes convert aflatoxins to the reactive 8,9-epoxide form (also referred to as aflatoxin-2,3 epoxide in the older literature), which is capable of binding to both DNA and proteins (Eaton and Groopman 1994). Mechanistically, it is known that the reactive aflatoxin epoxide binds to the N^7 position of guanines. Moreover, aflatoxin B_1-DNA adducts can result in GC to TA transversions, (Bennett and Klich 2003). Studies of liver cancer patients in Africa and China have shown that a mutation in the p53 tumor suppressor gene at codon 249 is associated with a G-to-T transversion (Bressace et al., 1991 and Hsu et al., 1991).

DNA adduct formation could be induced in rat tissues following oral administration of acrylamide as reported by Manière et al. (2005). Also, inhalation to low concentrations of 1,3-[2,3-[(14)C]-butadiene provoked DNA-adduct formation in tissues of rats and mice, (Booth et al., 2004). Such effect was reported by Pfohl-Leszkowicz et al. (1995) who studied the genotoxicity of zearalenone, an estrogenic mycotoxin in female mouse tissues.

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