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Pulsed Electric Field Technology for Checking Aflatoxin Production in Cultures and Corn Grains

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Exposure of aflatoxin-producing cultures of *Aspergillus flavus* to different pulsed electric field (PEF), reduced B_1 and G_1 toxin production. In recently inoculated cultures, continuous as well as 12 hrs exposure, every two days, to 50 Hz PEF resulted in the highest toxin decrease. Exposure of four-day old cultures to 0.50 and 50 Hz, as a daily treatment for 0.5-24 hr, reduced production by 75.53 and 82.75%, respectively. Increasing PEF from 100 to 400 Hz caused slight decrease in production, through the remarkable increase at 800 Hz. The highest amounts of aflatoxins B_1 and G_1 were associated with applying PEF strengths of 500 and 400 Hz, respectively. The multiple exposure at different PEF ranging from 800 to 500 Hz decreased production by 99.0 % maximum. It is worth to note that the B_1 aflatoxin was undetectable at different combined PEF strengths. Multiple exposure of yellow corn grains over 21 days to a combined treatment reduced the amount of aflatoxin, in either non-inoculated or in grains inoculated with *A. flavus*, compared to the check. Negligible changes were observed in protein and carbohydrates contents of the treated grains.

Key words: Aflatoxins, *Aspergillus flavus* and pulsed electric fields.

Yellow corn (*Zea mays* L.) is one of the most important grain crops all over the world. It is used mainly for animal, poultry feeding, and for human consumption in some developed countries. Aflatoxins B_1 and G_1 produced by certain strains of *Aspergillus flavus* were detected in rice, wheat, corn, soybean and sorghum (Hesseltine, 1965; Henry *et al.*, 1981 and Sauer and Tuite, 1986) and produced in semi-synthetic media (Davis *et al.*, 1966; Diener and Davis, 1986 and Abramson and Clear 1996). Zohri *et al.* (1995) studied the occurrence of aflatoxins and mould flora in 60 different maize snack samples in Egypt and found that all these samples were contaminated with aflatoxins B_1, B_2, G_1 and G_2 at concentrations ranging from 50 to 100 µg/kg. Eisa *et al.* (1996b) noticed that low graded corn grains (No. 3) had the highest amounts of both B_1 and B_2 aflatoxins compared with grade one. Saubois *et al.* (1998) reported that 4 out of 37 corn samples collected from Argentina were contaminated with aflatoxins in the range of 20 to 50 µg/kg. Thompson and Henke (2000) determined that aflatoxins were produced in maize grains regardless the type of the storage container, the time of storage and the climatic conditions.

The pulsed electric fields (PEFs) for inactivation of microorganisms is a promising non-thermal processing method to produce safe products (Wouters *et al.*, 1999) mainly in liquid foods (Calderon *et al.*, 1999). Evrendilek *et al.* (1999) recorded that the high voltage pulsed electric field (PEF) treatment proved to be a promising technology for the inactivation of *Escherichia coli* in apple juice. However, literature about using PEF against the plant pathogenic fungi is not available.

Dunn (1996) used the pulsed light (pure bright) process as short duration flashes of broad-spectrum (white) light to kill all exposed microorganisms, including protozoan oocysts. The intensity of each flash of light is about 20000 times the intensity of sunlight at the earth's surface. He added that multiple short duration, high intensity electric field pulses killed vegetative microorganisms in pumpable products. This pulsed electric field process can be applied at modest temperatures at which no appreciable thermal damage occurs and the original taste, colour, texture, and functionality of products can be retained.

Wouters *et al.* (1999) studied the effect of pulsed electric field (PEF) treatment and processing factors on the inactivation kinetics of *Listeria innocua* by using a pilot plant (PEF) unit with a flow rate of 200 l /hr. They found that the electric field strength, pulse length, number of pulses, and inlet temperature were the most significant process factors influencing the inactivation kinetics.

This study aimed to find out the possible use of pulsed electric field (PEF) as a safe technology for checking aflatoxins produced by *Aspergillus flavus* in both synthetic medium and in stored corn grains.

Materials and Methods

Application of the pulsed electric field (PEF) technique was carried out at the Faculty of Science, Biophysics Dept., Cairo Univ., Giza, Egypt using the GA-1230, 30 MHz synthesized ARBitarary, "Japan" wave generator (Fig. 1).

1- Effect of electric pulse field strength, numbers and time of exposure on in vitro aflatoxin production:

Erlenmeyer conical 100 ml flasks each containing 25 ml yeast extract sucrose (YES) medium were used (Park and Bullerman, 1981) for growing the aflatoxins producing isolate of *A. flavus* (Abou-El-Ella, 2002). Each flask was inoculated with 0.5 ml spore suspension of *A. flavus* (10^6 spore/ml) and incubated for 14 days at 25°C. The inoculated flasks were arranged in four groups and were exposed to PEF as follows:

A- Four days after inoculation; the cultures were exposed once for 1 hr to different pulse electric field strengths (0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40 and 50 Hz).

B- One hour after inoculation; the cultures were exposed to pulse electric field at strength of 50 Hz either continuously (along 24 hr daily) or for 0.5, 1, 3, 4, 12 hrs/day. The latter treatments were repeated every 2 days during incubation period.

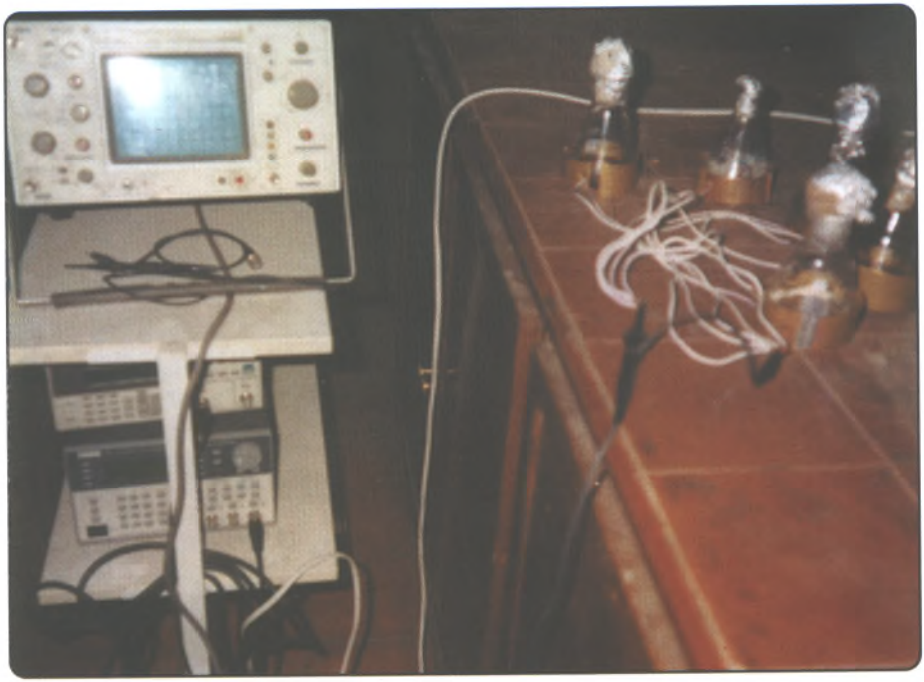

Fig. 1. MHz synthesized ARBitarary wave generator attached with flasks containing YES medium inoculated with *A. flavus* spores.

C- One hour after inoculation; the cultures were exposed once for 2 hrs to a relatively high pulse electric field strengths (100, 200, 300, 400, 500, 600, 700 and 800 Hz).

D- One hour after inoculation; the cultures were daily exposed for 4, 6 and 8 hrs to different combinations of pulse electric field strengths (700+800, 600+700+800, and 500+600+700+800 Hz). In this experiment, the exposure was done successively 2 hours for each particular pulse electric field strength.

In all the above mentioned treatments, the aflatoxins B_1 and G_1 were extracted from the cultural filtrates according to Davis *et al.* (1966) after 14 days from incubation and determined by comparing the unknown samples to quantitative standards on thin-layer-chromatography (TLC) as described below. The reduction rates in aflatoxins B_1 and G_1 production due to the use of PEF was compared with the control results (un-exposed) as follows.

$$\text{Reduction (\%)} = \frac{\text{Aflatoxin (µg/ml) in control - Aflatoxin (µg/ml) in treatment}}{\text{Aflatoxin (µg/ml) in control}} \times 100$$

2- Effect of PEF on total protein, carbohydrate and aflatoxin production in corn grains:

Imported yellow corn grain samples (grade 2) were scratched by shaking with sand for 1 min, disinfested by immersing in 5% sodium hypochlorite for 2 min, washed thoroughly with sterilized water and dried in a hot-air oven at 44°C for 24 hrs to reach a constant moisture content (11%) of the tested grains. Spore suspension was prepared from pure cultures of an aflatoxin producing isolate of *A. flavus* (Abou-El-Ella, 2002) grown for 21-days on PDA plates (9 cm). These plates were flooded with 15 ml of sterilized distilled water and brushed thoroughly for 1–2 min. The suspension was filtered through three layers of cheesecloth to remove the mycelial residues. Number of spores/ml. was counted and adjusted by using a Spencer haemocytometer to give $7000/10^4$ spores/ml. Spore suspension was inoculated to give approximately 3000-3500 spore/gram of corn grains as described by Eisa *et al.* (1996a).

Moisture content of corn grains was adjusted to the required moisture (25%) by adding calculated volumes of sterilized distilled water to the tested quantity of grains. The required volume of water needed for each moisture content was calculated according to the following equation as described in the approved method of the American Association of Cereal Chemists (A.O.A.C., 1990).

$$S = \frac{\text{Required moisture content} - \text{Initial moisture content}}{100 - \text{Required moisture content}} \times 100$$

Whereas: S= The volume of water required for 100 g of corn grains to reach (25%).

All samples were inoculated with spore suspension of *A. flavus* at the rate of 3000-3500 spore/g grains (Eisa *et al.*, 1996a). Aflatoxin production was determined in 100g of each sample. Another amount of grains was kept without inoculation to serve as control. Both inoculated and non-inoculated grains were exposed to combined PEF strengths including 800, 700, 600 and 500 Hz (2 hrs/day for each particular pulse electric field with total exposure time of 8 hrs/day) for successive 21 days.

Determination of aflatoxins:

Thin layer chromatography (TLC) was used for B_1, B_2, G_1 and G_2 determination in all treatments according to A.O.A.C. (1990).

Determination of total protein:

One gram from each sample of grains was digested using a mixture of concentrated sulphuric acid and hydrogen peroxide (40%). The total nitrogen was determined according to the standard official methods and the percentage of crude protein was then calculated according to A.O.A.C. (1990).

Determination of total carbohydrates:

Carbohydrates were determined using the phenol-sulphuric acid method described by Dubois *et al.* (1956).

Results

1- Effect of one hour single exposure to different PEF on aflatoxin producing cultures:

Exposure of 4-day-old cultures of *A. flavus* to different PEF strengths has markedly reduced G_1 and B_1 aflatoxins. The degree of B_1G_1 reduction was proportional to the strength of PEF ranging from 75.53% to 82.72% for the treatments 0.5 Hz and 50 Hz, respectively (Table 1). Noticeable fluctuation in G_1, however, was reported under the afore-mentioned conditions and the decrease in B_1 was greater than G_1.

Table 1. Effect of exposing 4-day-old cultures of *A. flavus* for 1 hour to different PEF strengths on their aflatoxin production

PEF (Hz)	Aflatoxin (µg/ml)			Reduction (%)
	B_1	G_1	Total $B_1 + G_1$	
0.5	16.04	15.16	31.20	75.53
1.0	16.00	15.20	31.20	75.53
2.0	16.40	14.40	30.80	75.84
4.0	15.60	14.60	30.20	76.31
6.0	14.80	15.20	30.00	76.47
8.0	14.80	15.60	30.40	76.16
10.0	14.40	15.20	29.60	76,78
12.0	14.00	14.80	28.80	77.41
14.0	13.60	15.04	28.64	77.54
16.0	14.60	13.96	28.56	77.60
18.0	13.60	14.80	28.40	77.73
20.0	13.20	14.84	28.04	78.00
30.0	12.40	14.80	27.20	78.67
40.0	12.00	13.20	25.20	80.24
50.0	10.00	12.00	22.00	82.75
Control	52.5	75.0	127.5	

2- Effect of multiple exposure of cultures to 50 Hz PEF for different durations:

Exposure of one hour-old culture of *A. flavus* to 50 Hz, followed by multiple exposure(s) at two-day intervals, greatly reduced aflatoxin production (Table 2). Exposure of culture for 12 or 24 hrs every 2 days caused the highest reduction in the total B_1G_1 being 93.16% and 93.10%, respectively.

3- Effect of high PEF on aflatoxin production by A. flavus:

Aflatoxin production has responded variably to 2-hrs exposure to high (100-800 Hz) pulse electric field strengths (Table 3). The percentage of reduction in aflatoxin was slightly decreased from 37.25% to 5.90 by increasing strength from 100 to 400 Hz. However, the percentage of reduction in aflatoxin production was increased again by elevating PEF from 500 Hz to 800 Hz. In this respect, the highest amounts of aflatoxins B_1 and G_1 were associated with PEF strengths of 500 and 400 Hz treatments as compared with the control treatment.

Table 2. Effect of multiple exposures of *A. flavus* cultures (one hour after inoculation) for different periods to pulsed electric field (50 Hz) on aflatoxin production

Exposure time* (hrs)	Aflatoxins (µg/ml)			Reduction (%)
	B_1	G_1	Total $B_1 + G_1$	
½	7.45	5.71	13. 16	89.63
1	6.25	5.00	11.25	91.18
2	4.05	5.50	9.55	92.51
3	3.00	6.25	9.25	92.75
4	2.88	6.04	8.92	93.00
12	4.80	3.92	8.72	93.16
24	3.92	4.40	8.32	93.10
Control	52.5	75.0	127.5	

* Cultures grown on YES medium were exposed repeatedly every 2 days along incubation period (14 days).

Table 3. Effect of exposing 1 hr *A. flavus* cultures for 2 hrs to different PEF strengths on aflatoxin production

PEF strengths * (Hz)/2 hrs	Aflatoxin (µg/ml)			Reduction (%)
	B_1	G_1	Total $B_1 + G_1$	
100	40.0	40	80.0	37.25
200	24.0	56	80.0	37.25
300	24.0	64	88.0	30.98
400	40.0	80	120.0	5.90
500	42.0	60	102.0	19.90
600	30.0	20.4	50.4	60.4
700	20.0	20.4	40.4	68.4
800	10.0	25.0	35.0	72.5
Control	52.5	75.0	127.5	

* Exposure to different PEF strengths was done by exposing the inoculating medium with *A. flavus* one time for 2 hrs only.

4- Effect of 1-hr A. flavus cultures exposure to different combined PEF:

Table (4) show the effect of increasing the daily exposure of *A. flavus* cultures to combined pulsed electric field strengths. The daily successive exposure to the combined PEF 800 + 700 + 600 + 500 Hz (2 hrs for each) has reduced the aflatoxin production by 99.0%. Daily exposure for 6 and 4 hrs to the combined PEF 800+700+600 Hz and 800+700 Hz reduced aflatoxin production by 98.0 and 95.1%, respectively. It is worthy to state that aflatoxin B_1 was not detected in YES cultures of *A. flavus* exposed to these combined PEFs.

Table 4. Effect of exposing 1 hr *A. flavus* cultures to different combined PEF on aflatoxin production

Combined PEF (Hz)*	Aflatoxin (µg/ml)			Reduction (%)
	B_1	G_1	Total $B_1 + G_1$	
1- 800+700	00.0	6.24	6.24	95.1
2- 800+700+600	00.0	2.50	2.50	98.0
3- 800+700+600+500	00.0	1.26	1.26	99.0
Control	52.0	75.0	127.5	

* Cultures were exposed 2 hrs daily for each particular PEF strength, *i.e.* total exposure time was 4, 6 and 8 hrs/day for treatments 1, 2 and 3, respectively.

5- Effect of exposing yellow corn grains on in vivo production of aflatoxins:

Data in Table (5) show that the daily exposure to 800, 700, 600, 500 Hz PEF successively (2 hrs for each) over 21 days period reduced the aflatoxins by 21.16% in non-infested yellow corn grains and 12.36% in yellow corn grains infested with *A. flavus*. The aflatoxins B_1, B_2, G_1 and G_2 were reduced by 10.0, 15.8, 9.35 and 14.9% in inoculated grains. The corresponding figures in the non-inoculated yellow corn grains were 0.0, 26.77, 25.89 and 16.58%, compared to the untreated controls.

Table 5. Effect of exposing yellow corn grains to combined PEF strengths (800+700+600+500 Hz)* for 8 hrs/day on aflatoxins production

Treatment	Aflatoxin (µg/100 g grains)				Total
	B_1	B_2	G_1	G_2	
Inoculated grains					
Exposed	92.46	109.58	171.20	136.98	510.22
Non-exposed (Control)	102.74	130.14	188.86	160.96	582.20
Reduction (%)	10.0	15.8	9.35	14.90	12.36
Non inoculated grains					
Exposed	27.4	60.19	126.90	125.69	340.18
Non-exposed (Control)	27.4	82.19	171.23	150.68	431.50
Reduction (%)	0.0	26.77	25.89	16.58	21.16

* Corn grains were exposed to each particular pulse electric field for 2 hrs daily along 21 successive days.

6- Effect of exposing imported yellow corn grains to combined PEF strengths (800+700+600+500 Hz) on protein and carbohydrate contents:

Data in Table (6) show crude protein and carbohydrate contents in corn grains as affected by the combined PEF treatment. The results showed that the crude protein was increased by 0.1 and 0.6% in inoculated exposed and non-exposed corn grains, respectively. In the non-inoculated corn grains the crude protein was increased by 0.3% in the non-exposed and contrary to the exposed corn grains. The carbohydrate content in inoculated corn grains was increased by 0.7 and 0.4% in exposed and non-exposed corn grains, respectively. The corresponding figure in the non-inoculated corn grains was 2.8% for both exposed and non-exposed corn grains.

Table 6. Effect of exposing yellow corn grains to combined PEF strengths on protein and carbohydrate contents

Treatment	Crude protein (%)	Change* (%)	Carbohydrate (%)	Change* (%)
Inoculated grains				
Exposed	8.4	0.1	72.9	0.7
Non-exposed (Control)	8.9	0.6	73.6	0.4
Non inoculated grains				
Exposed	8.3	0.0	73.0	2.8
Non-exposed (Control)	8.6	0.3	73.0	2.8
Non-exposed (Control)**	8.3		70.2	

* Compared with analysis of grains at zero time.
** Analyzed corn grain samples at zero time just before starting the experiment.

Discussion

Aspergillus flavus produces aflatoxins B_1 and G_1 in stored corn grains (Henry *et al.*, 1981 and Sauer and Tuite, 1986) and in semi-synthetic media (Davis *et al.*, 1966 and Diener & Davis 1986). Aflatoxins could be produced in stored maize grains regardless the type of storage container, time of storage and climatic conditions (Thompson & Henke, 2000).

Exposing yeast extract sucrose cultures (YES) of *A. flavus*, 1 hr or 4 days after-inoculation, to pulsed electric fields (PEF) caused remarkable decrease in production of aflatoxins B_1&G_1. Increasing PEF strength from 0.5 to 50 Hz has proportionally increased the degree of reduction from 75.53% to 82.75%. When recently inoculated cultures were exposed to PEF strength of 50 Hz for ½ -12 hrs every 2 days, reduction in aflatoxin production reached to 89.63 and 93.16%, respectively. The continuous exposure (24 hrs/day) decreased the amount of aflatoxins by 93.1% compared with the control.

When the recently inoculated YES medium was exposed once for 2 hours to relatively high PEF strengths (100-800 Hz), the aflatoxin production markedly fluctuated. The percentage of reduction was slightly decreased by increasing PEF strength from 100 to 400 Hz, then increased again by elevating PEF above 500 Hz. Such fluctuation may be influenced by the type of aflatoxin produced. In this respect, the highest amounts of aflatoxins B_1 and G_1 were found to be associated with applying 500 and 400 Hz PEF, respectively compared with the control treatment.

The daily exposure of the recently inoculated cultures for 8, 6 and 4 hrs to the combined PEF strengths 800+700+600+500 Hz, 800+700+600 Hz and 800+700 Hz, has reduced aflatoxin production by 99.0, 98.0 and 95.1%, respectively. These exposure treatments caused complete inhibition of B_1 aflatoxin production. These effects of PEF might be attributed to the matching between frequency of electric field currents and frequency of ionic metabolism of the microorganisms that causes

an enhancement in cellular activity, and increasing the toxin production as well. The effect of the interaction between the frequency of electric field current and the frequency of the ionic metabolism of the microorganisms, however, may explain the toxin decrease (Ali, 1998). Calderon *et al.* (1999) reported that pulsed electric field (PEF) is known to inactivate microorganisms by causing dielectric breakdown of the cell membrane, thus altering the function of the membrane as semi-permeable barrier. Jeyamkondan *et al.* (1999) reported also that the extent of damage of the cell membrane, whether visible in the form of pore or as loss of membrane function leads to the inactivation of the microorganisms. Inactivation of microorganisms exposed to high-voltage PEFs is related to the electromechanical instability of the cell membrane. Electric field strength and treatment are the two most important factors involved in PEF (Ali, 1998).

Exposing the imported yellow corn grains for 21 days (8 hrs/day) to PEF strengths 800+700+600+500 Hz (2 hrs for each particular PEF) reduced the amount of aflatoxins by 21.16% in non-inoculated and 12.36% in grains inoculated with *A. flavus*. The aflatoxins B_1, B_2, G_1 and G_2 were reduced by 10.0, 15.8, 9.35 and 14.9% in inoculated grains and 0.0, 26.77, 25.89 and 16.58%, respectively in non-inoculated yellow corn grains compared with respective controls (without exposure). Negligible changes were observed in protein and carbohydrate contents of the treated corn grains. Similar trend could be observed in the work of Grahi and Mark (1996). They pointed to the lethal effects of pulsed electric fields (PEF) on suspensions of various bacteria, yeast and spores in buffer solutions and liquid foodstuffs. They found that the counts of living-vegetative cells were reduced by PEF treatment meanwhile, the endo- and exo-spores were not inactivated or killed to any great extent. The killing of vegetative cell types depends on the electrical field strength of the pulses on the treatment time. The degree of destruction of food components (proteins, enzymes and vitamins, and the taste of foodstuffs due to PEF treatment was very low or negligible. Hence, PEF treatment is an excellent process for inactivation of microorganisms in acid and in thermo-sensitive media, but not for complete disintegration of microbial cells. Reina *et al.* (1998) indicated that PEF resulted in greater reduction of viable cells and the use of a high-voltage PEF is a promising technology for inactivation of food-borne pathogens.

In retrospect, it could be concluded that the strength of the pulsed electric field, number of exposures and duration are the main factors which affect the degree of aflatoxin destruction either in cultures of *A. flavus* or in the yellow corn grains. Further work is needed, however, to explain the exact influence of PEF(s) on the physiology of *A. flavus* in general and toxin production in particular.

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إستخدام موجات المجال الكهربى النابض كتكنولوجيا مبشرة لمنع إنتاج الأفلاتوكسينات فى مزارع الفطر أسبرجلس فلافس وفى حبوب الذرة المخزونة

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أدى تعريض بيئة مستخلص الخميرة والسكروز الملقحة بفطر الأسبرجلس فلافس (عزلة منتجة للأفلاتوكسينات) لجرعات مختلفة من المجال الكهربى النابض إلى إنخفاض واضح فى نسب الأفلاتوكسينات (B_1+G_1) المنتجة ، فقد أدى تعريض المزارع بعد ٤ يوم من تلقيحها لجرعات مختلفة من المجال الكهربى النابض تراوحت بين ٠,٥ –٥٠ هيرتز ولفترات تعريض يومى من ½ – ٢٤ ساعة لمدة ١٤ يوم متواصلة إلى خفض إنتاج الأفلاتوكسين بنسبة ٧٥,٥٣ و ٨٢,٧٥% على التوالى. كما أدى تعريض البيئة الملقحة (بعد ١ ساعة من التلقيح) لمدة ١٢ ساعة كل يومين وأيضا للتعريض اليومى المستمر لجرعة من المجال الكهربى النابض قدرها ٥٠ هيرتز إلى خفض كبير فى الكمية الكلية الأفلاتوكسينات (B_1+G_1) وصلت إلى ٩٣,١٦ ، ٩٣,١٠% على التوالى.

وقد تناقص الخفض فى إنتاج الأفلاتوكسين من ٣٧,٢٥– ٥,٩٠% بزيادة جرعات المجال الكهربى النابض من ١٠٠–٤٠٠ هيرتز ولكنه تزايد مرة أخرى ليصل ٧٢,٥% بزيادة جرعات المجال الكهربى النابض إلى ٨٠٠ هيرتز. كما وجد أن كفاءة جرعات المجال الكهربى النابض فى تحطيم أفلاتوكسينات الأسبرجلس ربما تعتمد على نوع الأفلاتوكسين حيث سجلت أعلى كميات للأفلاتوكسينات ب١ ، ج١ مصاحبة للمعاملة جرعات المجال الكهربى النابض عند ٥٠٠ و ٤٠٠ هيرتز على التوالى. وكان الخفض فى إنتاج الأفلاتوكسينات عاليا جدا كنتيجة للتعريض اليومى لمزارع الأسبرجلس فلافس بعد ساعة واحدة من التلقيح لجرعات متداخلة من المجال الكهربى النابض تراوحت بين ٥٠٠–٨٠٠ هيرتز ، حيث وجد أن التعرض لجرعات المجال الكهربى النابض المتداخلة (٨٠٠+٧٠٠+٦٠٠+٥٠٠ هيرتز) ، (٨٠٠+٧٠٠+٦٠٠هيرتز) ، (٨٠٠+٧٠٠هيرتز) قد خفضت إنتاج الأفلاتوكسينات بنسبة ٩٩,٠ ، ٩٨,٠ ، ٩٥,١% على التوالى.

وفى التجارب التطبيقية أدى التعريض اليومى لحبوب الذرة لمدة ثمانى ساعات لموجات المجال الكهربى النابض المتداخلة (٨٠٠+٧٠٠+٦٠٠+٥٠٠ هيرتز، ساعتان لكل واحدة) لمدة ٢١ يوم إلى خفض كمية الأفلاتوكسينات بنسبة ٢١,١٦ ، ١٢,٣ % فى حبوب الذرة الغير ملقحة و الملقحة بفطر الأسبرجلس فلافس (عزلة منتجة للأفلاتوكسينات) على التوالى مقارنة بالكنترول (الغير معرض). كما لوحظ أن التغيرات الحادثة فى محتوى حبوب الذرة المعاملة من البروتين و الكربوهيدرات كانت بسيطة جدا مقارنة بالكنترول (الغير معرض).