Amplitude Modulated Waves as a Promising Tool for Food Preservation

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A B S T R A C T

The present work was directed to study the possibility of using Amplitude Modulated Waves (AMW) in food preservation. The effect of AMW on the growth and, in vitro, production of aflatoxins by some fungal species isolated from stored kidney bean seeds was investigated. Eight fungal genera comprising 11 species were isolated from 4 months stored kidney bean seeds. Genus Aspergillus was the most dominant followed by Pencillium puberulum (RD. 62.8% and 19.2% of the total count, respectively). Aspergillus flavus (A. flavus) was the incomparable highly frequent species on the isolation plates. It constituted 35.6% of the total count. Only two isolates of A. flavus (I1 and I2) were found to produce aflatoxins in vitro, of the type B2, G1 and G2 in variable amounts. Exposure of the two isolates to amplitude modulated waves (AMW) for two hours at different frequencies inhibited spore germination and mycelia dry weight, irrespective of the wave frequency. The greatest inhibition was recorded at frequency of 10 Hz which completely suppressed the production of all types of aflatoxins. RAPD-PCR molecular analysis for aflatoxins producing fungi revealed relative changes in the position and number of some bands in DNA profile of both isolates, after treating with AMW at 10 Hz. Cluster analyses for PCR products revealed a decreased genetic similarity level between the two exposed isolates and their respective non-treated controls.

Key words: Food preservation, Biological effects, Electromagnetic waves, Asperagillus flavus, aflatoxins, Fungi.

Introduction

Fungal infection is one of the greatest problems facing storage of foods. Early in 1969, Christensen and Kaufman approved that seeds are subject for fungal contamination during storage which may destroy or at least devaluate them. Droby (2006) and Zhu (2006) estimated that about 20 up to 25% of fruits are damaged due to post-harvest handling and storage. Fungal infections of fruits may occur during growing, harvesting, handling, transport and storage (Bamidele et al., 2012). Furthermore, some of these fungi produce serious carcinogenic mycotoxins (Smith and Ross, 1991). One of these toxins is aflatoxins which are produced by certain species of Aspergillus like A. flavus and A. parasiticus (Palmgren and Ciegler, 1983; Samson and Frisvad, 1990; Truckess et al., 2002). Accordingly, destruction of
aflatoxigenic fungi in stored foods is an urged problem. This could be achieved through different methods such as preservatives (Ji et al., 2004), fungicides or essential oils (Jain and Jain, 2001) and gamma radiation (Shahhoseini, 1998). But the traditional methods of food preservation always have their own problems. Sometimes cause loss of sensitive nutrients, denaturation of proteins as well as change of structure, color and/or taste (Bamidele et al., 2012). Additionally, the formation of undesirable substances is possible (Lipiec et al., 2004). Therefore, developing new non-thermal safe methods for food preservation is not luxuriousness but it becomes a must.

Recently, the possible use of electromagnetic waves for inhibiting fungal growth have been investigated and proofed in many studies (Ramsted et al., 2000; Zhang et al., 2002; Rizk, 2003; Fadel et al., 2009; Bamidele et al., 2012; Ivona et al., 2015). Electromagnetic waves are non-ionizing radiation proved to affect some physiological activities of living organisms (Piskorz-Binczycka et al., 2003; Maxim et al., 2004; Aronsson et al., 2005). Such effects are found to be time, frequency and intensity dependent (Grahl and Markel, 1996; MacGregor et al., 2000; Bamidele et al., 2012).

The present study is one of the efforts devoted to investigate the possible use of Amplitude Modulated Waves (AMW) as a non-thermal safe technique for food preservation.

**Materials and Methods**

**Samples**

Kidney bean seeds (*Phaseouls vulgaris* L.), previously stored for four months were used in this study. Samples of four different cultivars (Nebraska, Giza 3, Giza 6 and Serbo) were obtained from agricultural research center, Giza, Egypt.

**Fungal isolation**

The isolation of seed born fungi was carried out according to Christensen (1957). The developing fungal colonies were counted then identified according to Raper and Fennell (1965); Ellis (1971); Barnett and Hunter (1987); Klich and Pitt (1988).

**Aflatoxins production**

**Qualitative estimation for aflatoxins production**

*Aspergillus flavus*, isolated from kidney bean seeds in the last procedure, were studied for aflatoxin production. The isolates were grown on SMKY agar medium, and then incubated for 10 days at temperature of 27 °C. The ability of the separate colonies to produce aflatoxins was examined by using ultraviolet light of wavelength 365 nm through checking the fluorescence around each colony. Only two isolates of *Aspergillus flavus* were found to be able to produce aflatoxins.

**Quantitative estimation for aflatoxins production**

Fungal discs (1 cm, 5 days old) of the aflatoxins producing isolates were used to inoculate flasks containing 50 mL SKMY medium, separately. Three replicates were prepared for each isolates, and then incubated at 27 °C for 10 days.

Aflatoxins were extracted according to Davis et al., (1966) from the filtrate. Standards of aflatoxins B1, B2, G1 and G2 (Sigma) were used to determine the type of aflatoxins produced by using thin layer
chromatography (TLC). While, the amount of aflatoxins produced was estimated using high performance thin layer chromatography (HPTLC) apparatus (Bio Doc Analysis, Germany).

**Exposure facility**

The two aflatoxins producing isolates of *A. flavus* were grown on Czapek-Dox’s agar medium for 5 days at temperature of 27 °C, then exposed to AMW with different frequencies (1, 5, 10, 15 and 20 Hz) for 2 hrs. The samples were exposed to the AMW through two parallel copper disk electrodes, each of diameter 8 cm. The modulating waveform was square and the carrier frequency was 10 MHz sine wave. The wave carrier was generated by a wave generator model AFG 310 manufactured by Sony Tektronics, Japan, and the modulating wave was generated by synthesized arbitrary generator type TTi TGA 1230. The amplitude of the wave carrier was 20 V and the modulating depth was ±2V (Fadel *et al.*., 2009).

**Spore germination**

Spores of both exposed and unexposed isolates were examined for germination using slides with central cavity. The exposed ones were examined directly after exposure to AMW. Spore suspension was prepared in sterile distilled water, then 0.1 mL of the suspension was introduced into the slides cavities (triplicates, 10 microscopic field per slide) and covered with a sterile edge greased cover glass. Then incubated at 28 °C for a suitable period of time and the germinated spores were counted and the percentage germination of spores was calculated and compared to that of the unexposed control.

**Mycelial dry weight**

Mycelia dry weight was determined using Czapek-Dox’s medium. Discs of diameters 1 cm from both exposed and unexposed fungi were inoculated into flasks containing 50 mL of the medium (triplicates for both samples). The flasks were incubated for 7 days at temperature of 27 °C. The mycelia, for both exposed and unexposed ones, were harvested, dried at 60 °C and their dry weight was determined and compared.

**Molecular analysis for aflatoxins producing isolates**

DNA Extraction: fungal discs of 1 cm in diameter, and 5 days old of both isolate of *A. flavus* just after exposure to AMW, as well as, unexposed (control) one were used to inoculate different flasks, each containing 50 mL of Czapel-Dox’s medium. Flasks were prepared in triplicates for each sample. The flasks were incubated for 5 days. The mycelia were separated and washed then DNA was extracted from 50 mg fresh mycelium using Qiagen Kit and dissolved in 100 μL buffer of PH 8.0 (Pieter *et al.*, 1995).

**Random amplified polymorphic DNA (RAPD) - PCR technique**

5 μL of the previously extracted genetic material were amplified using polymerase chain reaction (PCR) model UNII, Biometra, Germany (Pieter *et al.*, 1995). The PCR reaction mixture (Taq-DNA polymerase and dNTP) and the primer sequence (5’T[GTAGACCCGT]3’) were used for amplification according to the following PCR cycling conditions (45 cycles).
1- Denaturation at 95 °C for 1 minute followed by primer annealing at 36 °C for 1 minute.
2- Extension at 72 °C for 2 minutes and final extension at 72°C for 5 minutes, then, cooling at 4 °C.

**Gel electrophoresis:** The PCR products were electrophorated in 1% agarose gel, using WIDE-mini-sub cell GT, Bio-Rad electrophoresis and stained with ethidium bromide. The gel was scanned for bands using gel documentation program (Cairns and Murray, 1994) and the level of similarity was calculated through cluster analysis.

**Statistical analysis**

Analysis of variance was detected to evaluate the significance between means using SPSS statistical software (P < 0.01)

**Results and Discussion**

Four cultivars of kidney bean seeds (Nebraska, Giza 3, Giza 6 and Serbo) were examined for seed born fungi after storage of 4 months. Eight fungal genera with a total of 655 colonies/seed, comprising 11 species, were isolated from them (Table 1). The most prevalent species was Aspergillus, constituting 62.8 % of the total population, followed by Penicillium puberulum with 19.2 % of the total populations. *A. flavus* was the most dominant species in the 4 cultivars and recorded the highest relative density (RD 35.6% of the total count). Lower densities were observed for *A. niger*, *A. ochraceus* and *A. parasiticus* (RD 17.7, 6.9, and 2.6%, respectively). Alternaria alternate came next and it accounted for 5.6 % of the total count. Also Mucor hiemalis and Nigrospora sp. were isolated from kidney bean seeds, each with RD less than 5% of the total count. The other species, Epicoccum purpurascens, Chaetomium globosum and Trichothecium roseum were detected occasionally and all of them constituted 3.2 % of the isolations.

Fungal growth in stored seeds and grains is affected by water and temperature (Magan and Lacey, 1988) these fungi produce mycotoxins (Smith and Rose, 1991) which represent threats not only to vitality of seeds but also to the health of animals and humans feed on them. The prevalence of some members of Aspergilli and Penicillia in stored seeds and grains is well known worldwide (Smith and Ross, 1991; Abou El-Ella, 2002). Also, the most dominant *A. flavus* produces exudates which increase seed moisture content and thus improves the growth conditions for other species (Christensen, 1972).

From the table it is clear that *A. flavus* achieved the incomparable highest total count on the isolation plates. Therefore, it was chosen to be examined for in vitro production of aflatoxins. Screening of all isolates of *A. flavus* (233 isolates) for qualitative production of aflatoxins revealed that two isolates only (*A. flavus* I1 and *A. flavus* I2) produce aflatoxins of the types B2, G1 and G2 (Figure 1).

The total amount of aflatoxins produced by *A. flavus* I1 and I2 is illustrated in Table 2. *A. flavus* is well known to produce aflatoxins (Palmgren and Ciegler, 1983) and the variability in the type and quantity of these toxins among strains has been established (Klich and Pitt, 1988; Cotty, 1989; Abramson and Clear, 1996; Ji et al., 2004).

In the present study, fungal cultures of the aflatoxin producing isolates of *A. flavus* were exposed to AMW with different frequencies. All the applied frequencies exhibit a variable degree of inhibition for growth criteria, except at 1 Hz which showed non-significant effect (P < 0.01) on either percentage spore germination or mycelia dry
weight (Figures 2 and 3, respectively). The maximum inhibition has been observed when samples were exposed to AMW of frequency 10 Hz. However, no linear correlation could be observed between the frequency of the applied waves and the degree of inhibition occurred. Similar results were observed by Geveke and Brunkhorst (2003). The response of both isolates after exposure to AMW was nearly similar. Likely, electromagnetic waves suppressed the germination of the sclerotia for both Sclerotium rolfsii (Rizk, 2003) and Sclerotium cepivorum (Fadel et al., 2009), as well as, inhibit the mycelia growth (Rizk, 2003) while, AMW accelerated the spore germination and mycelia growth of Penicillium claviforme (Piskorz-Binczycka et al., 2003).

Additionally, electromagnetic waves induced great reduction in viable cells of microorganisms depending on the wave frequency, time of exposure and/or the exposed organism (Grahland Markl, 1996). Such reduction might be due to breakdown of cell membrane (Calderon-Miranda et al., 1999; Schoenbach et al., 2000; Dunne, 2000; Oshima and Sato, 2004; Aronsson et al., 2005).

It is clear from the given data that, the maximum effect of AMW, on both isolates of A. flavus, occurred at frequency of 10 Hz. Therefore, its effect on aflatoxins production was investigated. The data showed that, the ability of both isolates to produce aflatoxins was highly suppressed when exposed to AMW with frequency of 10 for 2 hrs (Figure 4), compared to that for unexposed ones (Figure 1). This was evident from the disappearance of the bands of all types of aflatoxins (B2, G1 and G2). Such response indicates that AMW with frequency of 10 Hz may resonate and interferes with physiological process involved in aflatoxins production.

Table 1 Total count and relative density (RD%) of fungi associated with four cultivars of kidney bean seeds after storage for 4 months.

| Fungal species | Kidney bean cultivars | Total count | RD% |
|----------------|-----------------------|-------------|-----|
|                | Nebraska | Giza 6 | Serbo | Giza 3 |             |
| Aspergillus spp. |          |         |       |       |             |
| A. flavus  | 43       | 63      | 48    | 79    | 233        | 35.6    |
| A. niger   | 35       | 58      | 0     | 23    | 116        | 17.7    |
| A. ochraceus | 11     | 21      | 3     | 10    | 45         | 6.9     |
| A. parasiticus | 0      | 13      | 2     | 2     | 17         | 2.6     |
| Penicillium puberulum | 43    | 10      | 46    | 27    | 126        | 19.2    |
| Alternaria alternate | 0     | 0       | 26    | 11    | 37         | 5.6     |
| Nigrospora sp | 1      | 10      | 10    | 8     | 29         | 4.5     |
| Epicoccum purpurascens | 0   | 0       | 0     | 2     | 2          | 0.3     |
| Chaetomium globosum | 0    | 8       | 0     | 0     | 8          | 1.2     |
| Mucor hiemalis | 0     | 13      | 2     | 16    | 31         | 4.7     |
| Trichotheceum roseum | 9     | 0       | 0     | 2     | 11         | 1.7     |
| Total         | 142     | 196     | 137   | 180   | 655        | 100     |
Table 2 Quantitative estimation for aflatoxin production (μg/50 mL medium) by the two isolates of A. flavus.

| Isolates   | Type of aflatoxin | Total |
|------------|-------------------|-------|
|            | B2                | G1    | G2    |       |
| A. flavus I<sub>1</sub> | 14.22             | 13.77 | 13.86 | 41.85 |
| A. flavus I<sub>2</sub> | 10.00             | 13.97 | 12.38 | 36.35 |

Fig. 1 Qualitative screening for aflatoxin production by two isolates of A. flavus. Lane 1: A. flavus I<sub>1</sub>, Lane 2: A. flavus I<sub>2</sub>, Lane 3: Aflatoxins standard.

Fig. 2 Effect of AMW with different frequencies on the percentage germination of A. flavus isolates.
Fig. 3 Effect of AMW with different frequencies on the mycelia dry weight (mg/flask) of  A. flavus  isolates

![Bar graph showing the effect of AMW on mycelia dry weight of A. flavus isolates.](image)

A. flavus I₁  
A. flavus I₂

Fig. 4 Aflatoxins production by  A. flavus I₁  (Lane 1) and  A. flavus I₂  (Lane 2) after exposure to AMW with frequency 10 Hz for two hs, as compared to standard (Lane S).
**Fig. 5** Gel electrophoresis of PCR Products of control (unexposed) isolates of *A. flavus* I1 (Lane 1) and *A. flavus* I2 (Lane 2) as compared to exposed ones, *A. flavus* I1 (Lane 3) and *A. flavus* I2 (Lane 4).

**Fig. 6** Genetic similarity levels between control (unexposed) isolates of *A. flavus* I1 (Lane 1) and *A. flavus* I2 (Lane 2) as compared to exposed ones, *A. flavus* I1 (Lane 3) and *A. flavus* I2 (Lane 4).

Similar effect was observed by Eisa *et al.*, (2002), when exposed corn grains inoculated with *A. flavus* to electromagnetic waves reduced, in situ, aflatoxins production without affecting grain quality. A similar inhibiting effect for AMW was observed by EL-Hag (2003), on his study on *Pseudomonas aeruginosa*. The study showed that AMW suppressed the toxins production by *Pseudomonas aeruginosa* when exposed to AMW of frequency of 0.5 Hz.
Molecular analysis for aflatoxin producing isolates, exposed to AMW of frequency 10 Hz, was carried out after DNA amplification by PCR technique (Figure 5). The results revealed relative changes in the position and number of some bands in the DNA profile of both isolates, which were almost similar. These observations may be denoting the interference of the AMW at this frequency with gene expression for aflatoxin production. Furthermore, genetic similarity level (Figure 6) between A. flavus I₁ and its comparable unexposed one was reduced to 92.17 % (7.83 % dissimilarity), whereas that of exposed A. flavus I₂ was reduced to 88.03% (11.97% dissimilarity).

In this connection, recent studies revealed that electromagnetic waves induce damage for cell structure and composition (Bamidele et al., 2012) starting from cell membrane (Bersani et al., 1997; Schoenbach et al., 2000) extending to the cytoplasm and nucleus (Hill, 1998). Exposure to extremely low frequencies EMW cannot induce direct damage to DNA, due to its low energy, but it may affect general and specific gene expression (Smiko and Mattson, 2004; Fadel et al., 2009). In addition, Fadel et al., (2009) suggested that this genetic effect may be attributed to bioelectric resonance interference resulted in the deterioration and/or alteration of the DNA genetic properties.

In conclusion, it may be concluded from the present study that, AMW may be a promising non-thermal safe technique for inactivation of harmful pathogenic moulds and their abilities to produce mycotoxins, aflatoxin included, in stored foods and grains instead of the health risks and other problems resulting from traditional methods of preservation. Future work on the possible effects of such waves on the viability of the treated grains is recommended.

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