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ANTIFUNGAL EFFECTS OF OZONATED WATER ON *ASPERGILLUS PARASITICUS*: A NEW APPROACH TO PREVENT WHEAT CONTAMINATION

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ABSTRACT

In Iran, wheat is one of the most important sources of diet. Unfortunately, it is vulnerable to contamination with fungi consisting of aflatoxigenic moulds. Since 1997, ozone has been recognized as an effective oxidant and disinfection agent. This study demonstrates the benefits derived from ozone in the inhibition of *Aspergillus parasiticus* growth and aflatoxin production in wheat. Wheat samples inoculated with *A. parasiticus* spore suspensions (10,10^2 and 10^4 spores/g of wheat) were treated with ozonated water at various concentrations of 0, 1, 2, 2.5 mg ozone/L at different temperatures. Fungal mycelium was weighed to evaluate the effect of ozone on *A. parasiticus* growth. Moreover, the rate of aflatoxin production was determined using high-performance liquid chromatography. Our results indicated that ozonated water can inhibit *A. parasiticus* growth as well as the accumulation of aflatoxins effectively. The best result was obtained when samples were treated with 2.5 mg/L of ozonated water and at fungal spore concentrations of 10 and 10^2 for inhibition of fungal growth and aflatoxin production, respectively ($P < 0.001$). In conclusion, the usage of ozonated water during wheat tempering is a promising strategy for inhibition of *A. parasiticus* growth and for reducing aflatoxin production.

PRACTICAL APPLICATION

The high incidence rate of cereal contamination by mycotoxins has been reported worldwide. Because of the carcinogenic effects of aflatoxins, various methods are used for inhibition of fungal growth and aflatoxin production. In contrast to different methods, which production of unfavorable residues is a problem, ozone decomposes to molecular oxygen rapidly and leaves no unwanted residue. Ozone is a safe, powerful disinfectant that is particularly suited for the food industry because of its ability to control biological growth of unwanted organisms without adding chemical by-products to the food being treated. Our findings indicated that usage of ozonated water instead of water in wheat tempering stage can possibly functioned as a method for inhibition of *Aspergillus parasiticus* growth and aflatoxins production in wheat and can play an important role in decreasing of cereal wastes and increasing of food safety.

INTRODUCTION

Globally, great economic losses occur when food spoilage is a consequence of moulds contamination. It has been documented that a large portion of the world's food products is wasted because of spoilage with fungi. Toxigenic strains of fungi can also have adverse effects on animal and human health by mycotoxin production (Freitas-Silva and Venâncio 2010; Muñoz *et al.* 2010; Imperato *et al.* 2011). Twenty-five percent of the world's grain supply is contaminated by mycotoxins (Sherif *et al.* 2009) of which aflatoxins (AFs) are responsible for a large part.

AFs are mutagenic, carcinogenic, teratogenic, nephrotoxic and immunosuppressive (Bluma and Etcheverry 2008; Köppen *et al.* 2010) secondary metabolites of fungi mainly produced by toxigenic strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Inan *et al.* 2007; Richard 2007). In addition, *A. tamarii* (Goto *et al.* 1996) and *A. pseudotamarii* (Ito *et al.* 2001) are also able to produce AF (Alberts *et al.* 2009). The major AFs are B_1, B_2, G_1 and G_2 (Inan *et al.* 2007). According to the International Agency of Research on Cancer, AFB_1 has been classified as group 1 of human carcinogens (Jalili *et al.* 2010). AFB_1 is the most toxic agent known in mammals and plays a role in etiology of liver cancer, notably among subjects who are hepatitis B virus surface antigen carriers (Imperato *et al.* 2011). The main source for human exposure to AFs are nuts, cereals (grains), dried fruits, figs, oilseeds, spices, cocoa beans and milk (Akbas and Ozdemir 2006; Khanafari *et al.* 2007; Wu *et al.* 2009). The Commission of European Communities established 4 μg/kg for total AFs (AFB_1, AFB_2, AFG_1, AFG_2) and 2 μg/kg for AFB_1 as the maximum level in cereals and all products derived from cereals (Škrbić *et al.* 2012). On the other hand, the Institute of Standards and Industrial Research of Iran established 15 μg/kg and 5 μg/kg for total AFs and AFB_1 as maximum acceptable levels in wheat (ISIRI 67/060 2002) which is the major part of the diet in Iran. Therefore, decontamination/detoxification methods of contaminated products urgently need to be applied. These methods can be classified as: chemical, physical (Hwang and Lee 2006; Basaran *et al.* 2008; Milićević *et al.* 2010) and biological (Dorner 2004; Wu *et al.* 2009) methods.

Chemical methods include the use of ammonia, sodium bisulfite, calcium hydroxide, formaldehyde, hydrogen peroxide (Basaran *et al.* 2008; Jalili *et al.* 2010) and ozone (Milićević *et al.* 2010). However, a problem of using chemical methods is the production of unfavorable toxic residues (Jalili *et al.* 2010). An advantage of applying ozone is that it is decomposed to molecular oxygen rapidly and leaves no unwanted residue (Kells *et al.* 2001; Naito and Takahara 2006; Inan *et al.* 2007; Tiwari *et al.* 2010).

Ozone is a strong oxidizing agent (Kells *et al.* 2001; Inanloo *et al.* 2011) and is effective in both gaseous and aqueous forms (Freitas-Silva and Venâncio 2010). The solubility and stability of ozone in water is dependent on some factors such as temperature, pH and purity of water (Freitas-Silva and Venâncio 2010). The antimicrobial properties of ozone have been documented previously (Guzel-Seydim *et al.* 2004; Rong *et al.* 2010).

There are no data available about use of ozonated water on inhibition of AFs in wheat. Thus, the main aim of current study was to investigate the effect of different ozonated water concentrations on inhibition of *A. parasiticus* growth and AFs production in wheat.

MATERIALS AND METHODS

Materials

Wheat samples were obtained from a commercial miller (Atlas, Tehran, Iran). An aflatoxigenic strain of *A. parasiticus* (ATCC 15517) was used to contaminate the wheat samples. All chemicals and media including standard AFs, B_1, B_2, G_1 and G_2 were purchased from Merck (Darmstadt, Germany).

Preparation of Samples, Inocula and Ozonated Water

Wheat samples were cleaned from foreign particles and other impurities (stones, foreign seeds, etc.) by hand. The initial moisture content of wheat was determined according to American Association of Cereal Chemists (AACC) method 44-15.02. Samples of 5 and 10 g of autoclaved, cleaned wheat were placed in 50 mL falcon centrifuge tubes for microbial and AFs analysis.

A. parasiticus was cultured on Sabouraud dextrose agar (Merck) plates for 72 h at 35C. Spores were harvested from 3-day-old cultures by adding sterile distilled water on the surface of a colony and gently scraping using a pipette tip. The number of spores was quantified using hemocytometer method. The spore concentrations were adjusted to obtain a final concentration of 10, 10^2 and 10^4 spores/g of wheat.

Ozonated water was obtained from a laboratory-scale ozone generator (COG-1A Model 6-5-11015 ARDA, France) that was equipped with an oxygen concentrator. The generator was capable of producing 0.8 g/h ozone. A volume of 250 mL of sterile distilled water in flask was connected to the ozone gas line. The ozone was bubbled through the water for about 45 min. To enhance the dissolved ozone concentration, flask was cooled in an ice bath during the procedure (Luo *et al.* 2012). During ozone generation, excess ozone was passed through 2% potassium iodide solution to prevent ozone from being released into the environment (Bialka and Demirci 2007). The concentration of the aqueous ozone was measured using the indigo

colorimetric method, which involves spectrophotometric measurement at 600 nm (APHA *et al.* 2005; Inanloo *et al.* 2011; Luo *et al.* 2012).

Experimental Design

The initial moisture content of wheat was 7.9% and was adjusted to ~17% by adding ozonated water and nonozonated water during tempering. Wheat samples were contaminated with the fungal spore concentrations mentioned earlier and incubated at 28 ± 2C (Diao *et al.* 2013) for 10 days. The samples were shaken each day to redistribute the inoculum. After 10 days, the samples were exposed to 0, 1, 2 and 2.5 mg ozone/L of ozonated water and then incubated at <20, 25 and 40C for 24 h. Three different controls were run along with the main tests, these included: contaminated wheat samples which were not ozonated, contaminated wheat samples which were ozonated and noncontaminated wheat samples which were ozonated.

Microbial Analysis

At the end of the incubation period, 10 mL of Sabouraud dextrose broth (Merck) medium was aseptically added to the 5-g wheat samples and was vortexed. The obtained supernatant was transferred to sterile Petri dishes and incubated at 28–30C for 3–5 days. To calculate the mycelial weight, fungal mycelium was filtered through preweighed filter papers, washed with distilled water, dried overnight at 55C and weighed for determination of fungal biomass.

AF Determination

High-performance liquid chromatography analyses were performed according to the methodology described by De Alencar *et al.* (2012) with some modifications. The wheat samples were milled and mixed to uniform consistency. Four grams of sample and 1 g of sodium chloride were added into falcon tubes. AF was extracted with 20 mL of 80% w/w methanol by shaking for 30 min at 2000 rpm (Heidolf multi Reax, Germany). Extracts were filtered through Whatman No. 4 filter paper. Six milliliters of the filtrate was collected in a 50 mL disposable centrifuge tube and added to 34 mL phosphate buffered saline pH 7.4, shaken vigorously and then passed through Glass Fiber Filter (GFF) filter paper. Diluted extract was passed through a Puri-Fast AFLA IAC immunoaffinity column (Libios, France) at a flow rate of 2–3 mL/min. Afterward the column was washed with 15 mL of phosphate buffer, and finally AF elution was performed with 1.25 mL of methanol and 1.75 mL of Milli-Q water (Merck KGaA, Darmstadt, Germany). The detection and quantification limit of the method was 0.1 and 0.3 μg/kg, respectively. Results are presented separately for AFB_1, AFB_2, AFG_1 and AFG_2. To determine the recoveries of analyzed AF, blank samples of wheat were spiked with 5 μg/kg AFB_1, 1 μg/kg AFB_2, 5 μg/kg AFG_1 and 1 μg/kg AFG_2.

Statistical Analysis

All experiments were performed in triplicate sets and results were represented as the mean values. Data were evaluated by analysis of variance using general linear models procedure of SPSS 20 and the significance was expressed at 5% significance level ($P < 0.05$). A Tukey's comparison was also performed to determine significant difference using a *P*-value less than or equal to 0.05.

RESULTS

Effect of Ozonated Water on *A. parasiticus* Growth and Mycelia Weight

Contaminated wheat samples, which were exposed to 10, 10^2 and 10^4 *A. parasiticus* spores, were treated with different ozonated water concentrations (0, 1, 2 and 2.5 mg/L) and then incubated at three different temperatures <20, 25 and 40C for 24 h. Microbiological analysis showed that tempering of the wheat samples with ozonated water reduced *A. parasiticus* growth in a dose-dependent manner ($P < 0.001$). Figure 1 indicates the inhibitory effect of different concentrations of ozonated water on *A. parasiticus* growth rate. Although the rate of fungal growth was

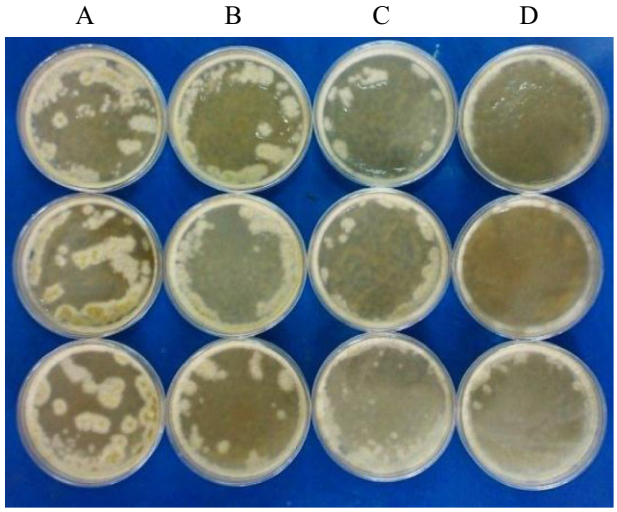

FIG. 1. EFFECT OF DIFFERENT CONCENTRATIONS OF OZONATED WATER ON *A. PARASITICUS* GROWTH (10^4 SPORES/G OF WHEAT). OZONATED WATER CONCENTRATION: (A) POSITIVE CONTROL, (B) 1 MG/L, (C) 2 MG/L, (D) 2.5 MG/L

TABLE 1. INFLUENCE OF DIFFERENT CONCENTRATIONS OF OZONATED WATER ON FUNGAL GROWTH OF *ASPERGILLUS PARASITICUS* (G/10 ML) IN WHEAT SAMPLES AT THREE TEMPERATURES AND DIFFERENT SPORE CONCENTRATIONS. VALUES ARE MEAN ± STANDARD DEVIATION OF THREE REPLICATIONS

Spore concentration (spores/g of wheat)	T (C)	Ozonated water (mg/L)			
		0	1	2	2.5
10	<20	0.048 ± 0.001	0.040 ± 0.002	0.038 ± 0.001	0.028 ± 0.003
	25	0.049 ± 0.004	0.041 ± 0.002	0.039 ± 0.003	0.031 ± 0.003
	40	0.050 ± 0.002	0.044 ± 0.002	0.039 ± 0.002	0.034 ± 0.003
10^2	<20	0.054 ± 0.003	0.042 ± 0.001	0.040 ± 0.003	0.036 ± 0.002
	25	0.055 ± 0.003	0.051 ± 0.001	0.043 ± 0.002	0.036 ± 0.002
	40	0.061 ± 0.002	0.054 ± 0.002	0.045 ± 0.002	0.037 ± 0.001
10^4	<20	0.096 ± 0.004	0.089 ± 0.003	0.088 ± 0.001	0.082 ± 0.003
	25	0.099 ± 0.003	0.091 ± 0.005	0.089 ± 0.002	0.086 ± 0.003
	40	0.104 ± 0.003	0.094 ± 0.001	0.090 ± 0.005	0.087 ± 0.002

reduced by the lowest concentration of ozonated water (1 mg/L), the best result was achieved when 2.5 mg/L of ozonated water was used.

Moreover, the obtained weight for fungal mycelia revealed the effectiveness of ozonated water on *A. parasiticus* growth (Tables 1 and 2). A significant decrease in mycelial weight occurred when samples were treated with the highest concentration of ozonated water. Table 3 indicates the percentage of reduction in mycelial weight of *A. parasiticus* exposed to different concentrations of ozonated water at three different temperatures. Considerable effect of temperature and primary spore concentration was observed in reducing fungal growth. Moreover, reduction in fungal growth increased with the maximum concentration of ozonated water at 10^2 spore concentration and <20C. Figures 2 and 3 indicate the effect of different ozonated water concentrations under various conditions.

Effect of Ozonated Water on AF Production

The effect of ozonated water on AF production at various concentrations and different temperatures is presented in Table 4. A significant reduction ($P < 0.001$) in AFs content was obtained by increasing the ozonated water concentration (Table 5). It should be noted that the amount of AFB_2 produced by fungi were below the detection limit of method in our study (Figure and Table 4).

	Degrees of freedom	Mean square	*F*	*P*
O_3	3	0.001461	236.15	<0.001
Different spore concentrations	2	0.028222	4562.77	<0.001
Different temperatures	2	0.000012	1.97	<0.001

TABLE 2. ANALYSIS OF VARIANCE OF THE EFFECT OF DIFFERENT CONCENTRATIONS OF OZONATED WATER (O_3) ON *ASPERGILLUS PARASITICUS* GROWTH AT DIFFERENT TEMPERATURES AND DIFFERENT SPORE CONCENTRATIONS

Spore concentration (spores/g of wheat)	T (C)	Mycelium weight reduction (%)			
		Ozonated water (mg/L)			
		0	1	2	2.5
10	<20	0.0	16.0	20.8	42.4
	25	0.0	16.2	21.6	37.2
	40	0.0	12.1	20.8	31.5
10^2	<20	0.0	22.2	32.5	48.8
	25	0.0	8.4	22.3	44.0
	40	0.0	10.4	26.4	44.0
10^4	<20	0.0	7.0	7.7	14.3
	25	0.0	7.7	9.8	12.8
	40	0.0	9.3	13.2	15.8

TABLE 3. PERCENTAGE OF MYCELIUM WEIGHT REDUCTION OF *ASPERGILLUS PARASITICUS* PRODUCED BY DIFFERENT SPORE CONCENTRATIONS IN WHEAT SAMPLES AT DIFFERENT CONCENTRATIONS OF OZONATED WATER AT THREE TEMPERATURES (T)

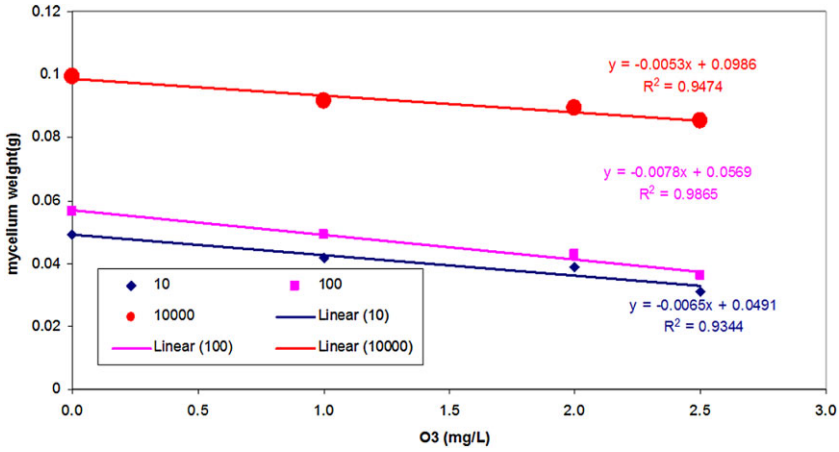

FIG. 2. EFFECT OF DIFFERENT CONCENTRATIONS OF OZONATED WATER (MG/L) AT DIFFERENT SPORE CONCENTRATIONS ON FUNGAL MYCELIUM WEIGHT. IT WAS DEMONSTRATED THAT THE SLOPES FOR ALL THREE LINES, WHICH REPRESENT THE CONCENTRATIONS OF FUNGAL SPORE, WAS APPROXIMATELY EQUAL. NEVERTHELESS, TO GET BEST RESULTS, LOW CONCENTRATIONS OF FUNGAL SPORES ARE RECOMMENDED

With regard to our results, the production of AFG_1 and AFG_2 were completely inhibited when samples were treated with 2 and 1 mg/L of ozonated water, respectively. However, AFB_1 production was not entirely inhibited at these ozonated water concentrations. The maximum reduction of AFB_1 production was obtained at 2.5 mg/L by which 27.4, 34.1 and 40% of the toxin production was inhibited at <20, 25 and 40C, respectively. The results indicated that two-order polynomial regression model was fitted to all response variables for percentage of AFB_1, AFG_1 and AFG_2 reduction as shown in Table 6.

DISCUSSION

In the present study, we took advantage of the antifungal feature of ozone in order to improve the quality of wheat which is regarded as the main part of the diet in many countries including Iran. Logically, the best result was obtained when the minimum amount of fungal spores was applied. It was revealed that using ozonated water at a concentration of 2.5 mg/L had a significant inhibitory effect on fungal growth rate at temperatures below 20C. Nevertheless, fungal spore concentration is a more important influential factor than temperature variation. In addition, our results were in agreement with other researchers. De Alencar *et al.* (2012) evaluated the fungicidal and detoxifying effects of ozone on AFs in peanuts. They found the growth rate of *A. flavus* and *A. parasiticus* decreased significantly ($P < 0.05$) by increasing the ozone concentration and exposure period. Accordingly, fungal growth was inhibited at a concentration of 21 mg/L during 96 h of incubation period (De Alencar *et al.* 2012). In 2006, Wu *et al.* showed that 96.9% of the fungal spores were inactivated by applying 0.33 mg of ozone/g wheat/min in 5 min (Wu *et al.* 2006). Kells *et al.* (2001) also found that in a period of 3 days, tempering of 8.9 tons of maize with 50 ppm of ozone reduced 63% of *A. parasiticus* contamination level on the kernel surface (Kells *et al.* 2001).

Not only does ozonated water have an inhibitory effect on fungal growth, but also the rate of AF production was considerably reduced by applying ozonated water to wheat samples. According to our results, AFG_1 and AFG_2 were

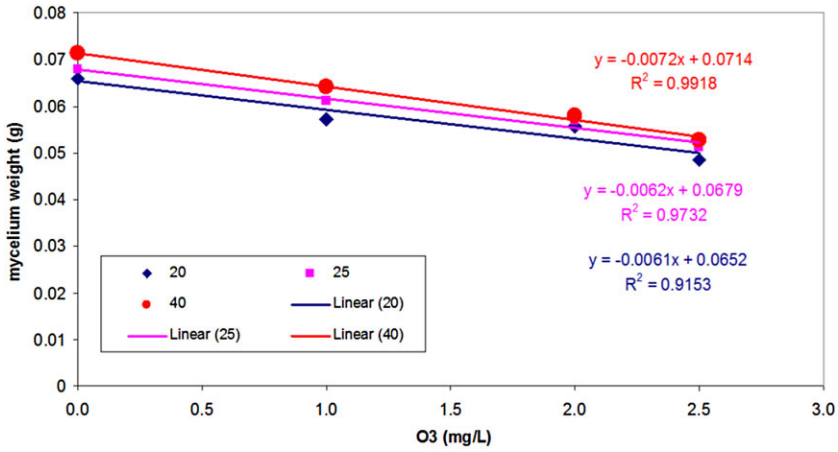

FIG. 3. EFFECT OF DIFFERENT CONCENTRATIONS OF OZONATED WATER (MG/L) AND DIFFERENT TEMPERATURES ON MYCELIUM WEIGHT OF FUNGI. IT WAS DEMONSTRATED THAT THE SLOPES FOR ALL THREE LINES, WHICH REPRESENT THE TEMPERATURE, WAS APPROXIMATELY EQUAL AND THAT ENVIRONMENTAL FACTOR HAS NO CONSIDERABLE EFFECT ON FUNGAL GROWTH RATE

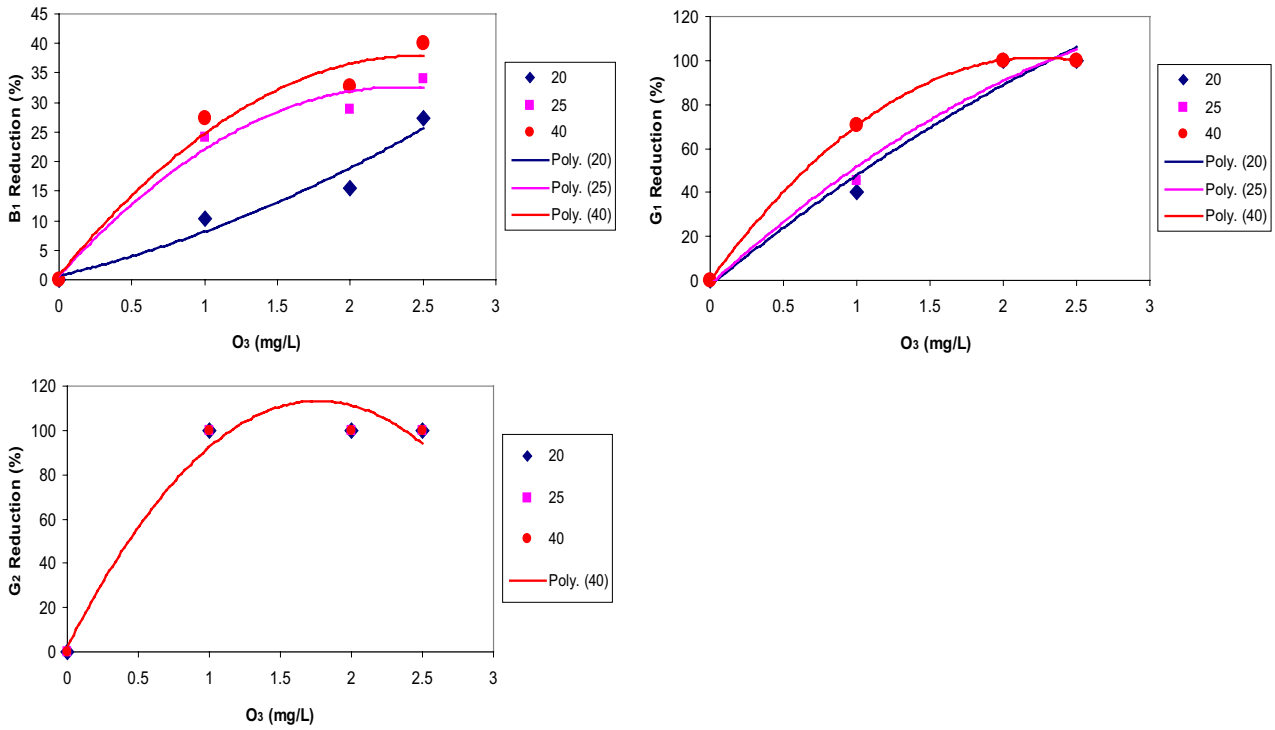

FIG. 4. PERCENTAGE OF AFLATOXINS REDUCTION PRODUCED BY *ASPERGILLUS PARASITICUS* IN WHEAT SAMPLES AT DIFFERENT CONCENTRATIONS OF OZONATED WATER AND DIFFERENT TEMPERATURES AS WELL

Incubation temperature (C)	Dose of O_3	AFB_1 (µg/kg)	AFB_2 (µg/kg)	AFG_1 (µg/kg)	AFG_2 (µg/kg)
<20	0	1.35 ± 0.05	ND	0.89 ± 0.04	0.51 ± 0.03
	1	1.21 ± 0.06	ND	0.53 ± 0.02	ND
	2	1.14 ± 0.05	ND	ND	ND
	2.5	0.98 ± 0.01	ND	ND	ND
25	0	1.70 ± 0.06	ND	1.76 ± 0.04	0.44 ± 0.03
	1	1.29 ± 0.03	ND	0.96 ± 0.01	ND
	2	1.21 ± 0.02	ND	ND	ND
	2.5	1.12 ± 0.02	ND	ND	ND
40	0	1.65 ± 0.05	ND	2.10 ± 0.10	0.48 ± 0.01
	1	1.20 ± 0.01	ND	0.62 ± 0.03	ND
	2	1.11 ± 0.04	ND	ND	ND
	2.5	0.99 ± 0.02	ND	ND	ND

ND, Not detected.

TABLE 4. INFLUENCE OF DIFFERENT CONCENTRATIONS OF OZONATED WATER ON DIFFERENT AFLATOXIN (AF) CONCENTRATIONS AT THREE TEMPERATURES (VALUES ARE MEAN ± STANDARD DEVIATION OF THREE REPLICATIONS)

		AFB_1			AFB_2			AFG_1			AFG_2		
	df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
O_3	3	1.4	317.7	<0.001	0	0	0	5.1	4209.8	<0.001	0.5	3879.3	<0.001
T	2	0.1	50.7	<0.001	0	0	0	0.4	349.8	<0.001	0.0	7.74	<0.001

df, degrees of freedom; MS, mean square.

TABLE 5. ANALYSIS OF VARIANCE OF THE EFFECT OF DIFFERENT CONCENTRATIONS (MG/L) OF OZONATED WATER (O_3) ON DIFFERENT AFLATOXIN (AF) CONCENTRATIONS (MG/KG) AT DIFFERENT TEMPERATURES (T)

TABLE 6. ADJUSTED REGRESSION EQUATIONS REFERRING TO AFLATOXIN B_1 REDUCTION IN WHEAT EXPOSED TO OZONATED WATER AT DIFFERENT CONCENTRATIONS AT DIFFERENT INCUBATION TEMPERATURES

Aflatoxin	Incubation temperature	Adjusted equation	R^2
B_1	<20	$y = 1.6862x^2 + 5.7566x + 0.6756$	0.95
	25	$y = -5.8025x^2 + 27.249x + 0.6163$	0.98
	40	$y = -6.0484x^2 + 29.995x + 0.7675$	0.97
G_1	<20	$y = -4.5565x^2 + 54.658x - 2.2274$	0.97
	25	$y = -7.1722x^2 + 60.644x - 1.8502$	0.98
	40	$y = -20.249x^2 + 90.57x + 0.0359$	1.00
G_2	<20	$y = -35.678x^2 + 125.88x + 2.2613$	0.97
	25	$y = -35.678x^2 + 125.88x + 2.2613$	0.97
	40	$y = -35.678x^2 + 125.88x + 2.2613$	0.97

more susceptible to ozone rather than AFB_1. AFG_1 and AFG_2 were totally inhibited using 2 and 1 mg/L of ozonated water. But AFB_1, however, was not completely inhibited even by using the highest concentration of ozonated water. The toxin production amount was nevertheless reduced. It seems that the most influential factor in AF reduction is only ozone concentration and temperature does not have a notable effect on AF reduction. In results similar to ours, Akbas and Ozdemir (2006); Öztekin *et al.* (2006); Inan *et al.* (2007); De Alencar *et al.* (2012); El-Desouky (2012) reported that reduction of AFB_1 levels in food crops (wheat, peanut, red pepper, dried figs and pistachio, respectively) was positively correlated with increases in the applied concentration of gaseous ozone (Akbas and Ozdemir 2006; Öztekin *et al.* 2006; Inan *et al.* 2007; De Alencar *et al.* 2012; El-Desouky 2012). Also, gaseous ozone and mild-heat treatment was used for reduction of AFs in peanut kernels and flour (Proctor *et al.* 2004).

Our results were in contrast with those reported by Mckenzie *et al.* in 1997. Accordingly, AFB_2 and AFG_2 were more resistant than AFB_1 and AFG_1 against ozone. The reason may be due to lower amounts of AFG_1 and AFG_2 in this study compared with the results reported by McKenzie *et al.* (1997). The efficacy of ozone treatment is influenced by ozone stability, solubility and reactivity that are temperature-dependent factors. Ozone reactivity is directly related to temperature but the solubility and stability are inversely related to this factor. However, the overall influence of these factors may compensate each other. Thus, different results may be obtained in different temperature ranges and with different systems (Ölmez and Akbas 2009). In conclusion, our results indicated that ozonated water can possibly be used as a method for inhibition of *A. parasiticus* growth in wheat. In additional, ozonated water caused AFs reduction. Future researches are needed to be conducted as to the effect of ozonated water on other mycotoxins. Also, higher concentrations of ozonated water are recommended to be evaluated for mycotoxins inhibition.

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