Food Sci. Biotechnol. 23(2): 661-669 (2014)
DOI 10.1007/s10068-014-0090-8

RESEARCH ARTICLE

The Antifungal Mechanism of Electrolyzed Oxidizing Water against *Aspergillus flavus*

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Received June 1, 2013; revised September 20, 2013; accepted September 25, 2013; published online April 30, 2014

Abstract Fungicidal electrolyzed oxidizing waters (EOW) include neutralized electrolyzed oxidizing water (NEW) and acidic electrolyzed oxidizing water (AcEW). These forms were evaluated against *Aspergillus flavus* conidia (spores) and mycelia. EOW possessed strong antifungal activities against *A. flavus* conidia and mycelia due to destruction of the cellular structures of the *A. flavus* conidium and mycelium. K^+ and Mg^{2+} leakage results from damage to the normal cellular functions of *A. flavus* conidia and mycelia. Among the physicochemical parameters of EOW, the available chlorine concentration (ACC) is primarily responsible for the EOW antifungal activity. *A. flavus* can be eliminated by use of AcEW and NEW as a new, alternative method of fungal control that is superior to some current physical methods and to synthetic chemical fungicides.

Keywords: electrolyzed oxidizing water (EOW), *Aspergillus flavus,* ionic leakage, available chlorine concentration (ACC)

Introduction

Growth of fungi in agricultural products causes spoilage and results in reductions in quality and quantity. Among the fungal species, *Aspergillus flavus* is of concern due to a toxigenic potential for producing aflatoxin in raw agricultural materials and derived products (1,2). In nature, *A. flavus* is capable of growing on many nutrient sources. It can infect corn, peanuts, maize, cotton, and nut trees. The fungus can often be seen sporulated on injured seeds, such as peanut and maize kernels (3). It also can be pathogenic to several plant and animal species, including humans and domestic animals. Furthermore, *A. flavus* not causes teratogenicity and carcinogenicity in organisms, but is also responsible for liver cancer in humans (4,5). *A. flavus* is also the second leading cause of aspergillosis in humans. Patients infected with *A. flavus* often have reduced or compromised immune systems.

To improve public health, controlling the processes of fungal growth and lowering the risk of *A. flavus* infection should be a top priority. Currently, a number of chemical fungicides and preservatives on the market have been linked to carcinogenic and teratogenic conditions, as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical additives and demand safe and socially more acceptable preservatives (6). The exploration for safe antifungal agents for raw agricultural materials and derived products is receiving increasing attention (7).

Previous reports have suggested that electrolyzed oxidizing water (EOW) has an antibacterial action. The main advantage of EOW is safety. When EOW contacts organic matter, or is diluted by tap water or reverse osmosis water, it reverts to ordinary water. Thus, there is a reduction in adverse effects on plant growth and the environment. EOW can be regarded as a type of new sanitizer (8-11). The 2 types of EOW that currently exist include neutralized electrolyzed oxidizing water (NEW) and acidic electrolyzed oxidizing water (AcEW). AcEW is the most investigated form due to special physicochemical properties, including a low pH (<3.0), a high oxidation-reduction potential (ORP, >1,000

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mV), a high dissolved oxygen content, and an available chlorine concentration (ACC). It has been used extensively for sanitation and quality control. Recently, research has also focused on NEW for inactivation of microbes (12-15). NEW is produced by electrolysis of a diluted hydrochloric acid solution in a non-membrane electrolytic cell (16) at a pH ranging from 5.0 to 6.5, an ORP of 800-900 mV, a high dissolved oxygen content, and ACC value.

Available information on evaluation of the EOW antifungal activity against *A. flavus* is lacking. The objectives of this study were to (i) to evaluate the fungicidal activities *of* EOW (NEW and AcEW) against *A. flavus* conidia and growth inhibition of *A. flavus* mycelia; (ii) to identify the main factors in EOW that account for antifungal activities; (iii) to investigate the reasons for main factors fungicidal activities against *A. flavus* conidia (spore) formation and mycelial growth.

Materials and Methods

***A. flavus* strain** *A. flavus* was isolated from peanut seeds (Species number: CA108). The fungus was identified using the Microbial Identification System according to standard morphological methods (17). Fungal cultures were maintained in Czapek medium (CZ, B.R. Grade; Shuang Xuan Microorganism Culture Medium Product Factory, Beijing, China) at 4ºC. Pathogenicity testing of *A. flavus* was performed by wiping suspensions of fungal conidia on the red skin of peanuts (CA108).

Preparation of an *A. flavus* conidia suspension *A. flavus* was planted into potato dextrose agar (PDA) (B.R. Grade; Aoboxing Biotech Co., Ltd., Beijing, China). The fungus was harvested from 2 to 3 day cultures at 30ºC. Fungal cultures were flooded with 10 mL of 0.5% sterile Tween 20 (R.T. Grade; Biodee Co., Ltd., Beijing, China) in distilled water (v/v). A suspension was obtained by slightly shaking prior to centrifugation (TGL-16C; Anting Scientific Instrument Co., Ltd., Shanghai, China) at 1,000×*g* for 5 min. The supernatant was discarded and the pellet was re-suspended in 10 mL of sterile distilled water (containing 0.5% Tween 20). The concentration of *A. flavus* conidia was measured and adjusted to 7.3-7.5 $\log_{10}$ conidia/mL using appropriate dilution with sterile distilled water.

Preparation of electrolyzed oxidizing water NEW and AcEW versions of EOW were prepared using two different generators. NEW was prepared using the NEW non-membrane electrolytic cell generator, model AQUACIDO NDX-250KMS (OSG Company Ltd., Osaka, Japan). AcEW was prepared using the membrane electrolytic cell model CE-7001AcEW generator (Sai Ai Environmental Protection and Technology Development Company Ltd., Guangzhou, China). AcEW and NEW were obtained from electrolysis of 8.2 mM NaCl and 8.2 mM HCl, respectively, for 15 min. Both types of EOW were used within 3 to 4 h after production. (18-21).

Three physicochemical parameters of NEW and AcEW pH, ORP and ACC values were verified and are shown in Table 1, compared with tap water (TW) and distilled water (DW). The pH and ORP values were measured using a 510M-01 multi-parameter measuring meter (Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA). The EOW available chlorine content (ACC) was measured using iodometric titration (22) in which NEW and AcEW were diluted in distilled water (23±3ºC) to obtain different chlorine concentrations.

Screening for fungicidal efficacy against *A. flavus* conidia Antifungal activity tests were carried out, The physicochemical properties of pH and ORP values of NEW and AcEW with different ACC values were measured at the same time. The ACC levels of NEW and AcEW were 30, 60, and 90 mg/L. Each treatment was carried out at 23±2ºC. A total of 100 μL of a conidia suspension was individually combined with 900 μL of either NEW or AcEW and maintained for 30 s with different ACC levels (30, 60, and 90 mg/L) for a total of 120 s. Reactions were stopped by addition of 9 mL of freshly prepared, filter-sterilized, pH 7.2 neutral buffer (KH_2PO_4 34.0 g/L; NaOH 7.0 g/L) (10). The amount of conidia surviving in the solution was determined using serial dilution in 9 mL of sterile neutral buffer, followed by direct plating of 0.1 mL of each dilution on Rose Bengal medium (B.R. Grade; Aoboxing). Plates were incubated at 30ºC for 48 to 72 h. Antifungal activities were evaluated by measuring the number of conidia survivors using the dilution plate counting method.

Table 1. Physicochemical parameters of different water types[1)]

	AcEW	NEW	Tap water[2)]	Distilled water
pH	2.2±0.1	6.0±0.2	7.1±0.3	6.2±0.2
ORP (mV)	1146±2	845±7	426±3	564±6
ACC (mg/L)	161.6±1.3	114.3±1.0	ND[3)]	ND

[1)] Values are mean±standard deviation (SD) (n=5).
[2)]Tap water was the drinking water at China Agricultural University. The chlorine residue was approximately 0.1 mg/L.
[3)]ND, no detected chlorine using the iodometric titration method

Determination of wet and dry mycelial weights NEW and AcEW (20 mL) with 30, 60, and 90 mg/L ACC levels were mixed with 20 mL of a fungal liquid culture medium (FLM) in an Erlenmeyer flask (B.R. Grade; Land Bridge Technology Co., Ltd., Beijing, China). Final ACC levels of NEW and AcEW in the liquid medium were 15, 30, and 45 mg/L. The conidial suspension (100 μL) was then added to the flasks. The FLM medium with only 20 mL of distilled water plus the fungal suspension was used as a control. Flasks were incubated with agitation (150 rpm) in an incubator (HZQ-F160; Dong Lian Electronic and Technology Development Co., Ltd., Harbin, China) at 30±2°C until strong mycelia growth was obtained after 72 h. Fungal mycelia were autoclaved (YXQ-LS-SM; Bo Xun Apparatus Co., Ltd., Shanghai, China) at 121°C for 30 min prior to harvest, followed by washing twice with distilled sterile water. After measuring the wet weight, mycelia were dried at 80°C. The dry weight of mycelia was determined.

Evaluation the roles of pH, ORP, and ACC as antifungal against against *A. flavus* A series of solutions was prepared to evaluate the effects of pH, ORP, and ACC on fungal growth of *A. flavus*. Solution 1 (S1) was AcEW with the physicochemical parameters shown in Table 1. Solution 2 (S2) was AcEW and was stored for 48 h at ambient temperatures after opening. The available chlorine content was gradually restored to the original state due to loss of the oxidizing ability after 48 h. Solution 3 (S3) was NEW with the physicochemical parameters shown in Table 1. Solution 4 (S4) was 0.1 M $Na_2S_2O_3$. Solution 5 (S5) was 0.2 M Na_2HPO_4 produced via electrolysis. There was no detectable available chlorine content in solutions S4 and S5 after the electrolysis procedure. Solution 6 (S6) was AcEW adjusted using 0.5 M NaOH. Solution 7 (S7) was a NaClO solution adjusted using 0.5 M NaOH. The physicochemical parameters of the solutions are shown in Table 2. *A. flavus* conidia suspensions (100 μL) were individually combined with 900 μL of different solutions, then maintained for 60 s. Distilled water without available chlorine was used as a control. Antifungal activities were evaluated by measuring the number of surviving conidia following the method of dilution plate counting method.

Ultraviolet spectroscopy analysis of different waters Using distilled water as reference for 100% transmittance, the absorption peak of available chlorine ionic form were measured for AcEW and NEW with ultraviolet scanning in the range of 200 to 380 nm. The values of the NaClO dilute solution absorption peak of available chlorine ionic compounds in multi-form (at pH values from 2 to 10 with intervals of 2 using 0.5 M HCl and NaOH individually and combined) were measured with ultraviolet scanning in the range from 200 to 380 nm.

Scanning electron microscopy of conidia treated with NEW and AcEW Conidia were suspended in 0.5% NaCl and 0.5% Tween 20 solutions and adjusted to 10^{6-7} conidia/mL. Each conidia suspension was centrifuged (TGL-16C; Anting Scientific Instrument Co., Ltd.) at 302×*g* for 5 min. The supernatant is removed by centrifugation, precipitate of the tube were fixed using 2.5% glutaraldehyde (TAAB), and then dehydrated in a graded ethanol series. Critical point drying with liquid CO_2 was performed on the samples before they were coated with gold-palladium and observed under a scanning electron microscope (23) (S-3400N; Hitachi, Tokyo, Japan).

Analysis the ionic permeability of conidia and mycelium by atomic absorption spectrophotometer (AAS) The ionic permselectivity of conidia and mycelia were evaluated for fungicidal activities in NEW and AcEW. *A. flavus* conidia and mycelia suspensions were prepared and adjusted using appropriate dilution to 7.5 $\log_{10}$ conidia/mL with sterile distilled water.

NEW and AcEW with ACC levels of 60 mg/L were prepared. Suspensions with 8.2 mM NaCl and 8.2 mM HCl were used as controls for the AcEW treatment group and the NEW treatment group, respectively. Amounts of 4 mL of NEW and AcEW were added to the suspensions as treatments. Conidia were centrifuged (Allegra X-15R; Beckman Coulter, Inc., Fullerton, CA, USA) at 3,356×*g* for 5 min. The supernatant was analyzed for concentrations of K^+ and Mg^{2+} using AAS (SOLAAR M6; Thermo, Waltham, MA, USA) (24,25). All reagents were of analytical grade. Water (resistance 18 M/cm) used in preparation of standard solutions. Samples were obtained using a Millipore Milli-Q-System (Millipore, Medford, MA, USA).

Statistical analysis Analysis of variance and Duncan's multiple range tests (at $p<0.05$) were performed to determine statistical differences and to discriminate between means (26). Values represented the means of two different tests with triplicate treatments per experiment. All analysis was performed using SAS statistical software (SAS Institute, Cary, NC, USA).

Results and Discussion

Antifungal activity of AcEW and NEW in pure cultures as a function of water properties Differences were observed between the antifungal activities of AcEW and NEW against *A. flavus* when pure cultures were subjected to different ACC levels during a 2 min treatment. Both AcEW and NEW significantly inhibited *A. flavus* compared with none treatment group ($p<0.05$). For the low ACC concentration test group (30 mg/L), the surviving fungal

Table 2. Evaluation of the roles of pH, ORP, and ACC in a series of antifungal solutions against *A. flavus*[1)]

Treantment	pH	ORP (mv)	ACC (mg/L)
Control	6.1±0.2[d2)]	520±8[e]	0
Tap water	7.7±0.1[c]	452±3[f]	0
AcEW (S1)	2.52±0.08[f]	1019±36[a]	30.5±1.2[b]
Oxydic AcEW (S2)	2.5±0.3[f]	1010±6[a]	10.2±0.3[c]
NEW (S3)	5.8±0.1[d]	886±13[bc]	31.8±3.1[b]
Electrolysis $Na_2S_2O_3$ (S4)	6.0±0.3[d]	873±6[c]	0
Electrolysis Na_2HPO_4 (S5)	2.5±0.1[f]	561±26[d]	0
AcEW adjusted by NaOH (S6)	4.1±0.2[e]	902±12[b]	31.8±3.1[b]
NaClO adjusted by NaOH (S7)	10.0±0.8[b]	538±8[de]	319.9±4.8[a]

[1)]Treated with distilled water taken as a control. Tap water was the drinking water come from China Agricultural University.
[2)a-f]Means sharing a different superscript are significantly different (p<0.01).

population was significantly decreased in the first 30 s of treatment with AcEW none treatment group (p<0.05), and then remained constant until 120 s had elapsed with a slight decrease in the surviving population near the end. The fungus was completely controlled by NEW after 90 s. For the mid-concentration (60 mg/L) and high (90 mg/L) ACC test groups, no surviving fungal population was detected in solutions treated with either AcEW or NEW (Table 2).

Effects of AcEW and NEW on inhibition of *A. flavus* mycelial growth The average wet and dry mycelial weights of *A. flavus* in a liquid medium with addition of EOW at different concentrations are shown in Fig. 1. Compared with the control, both AcEW and NEW significantly reduced the wet and dry weights of *A. flavus* mycelia in all cases compared with control (p<0.05). High ACC levels resulted in reduced wet and dry weights of *A. flavus* mycelia. The effect of NEW on reducing wet and dry mycelial weights was stronger than the effect of AcEW. There is significant results on reducing dry mycelial weights (p<0.05). Non-significant results should not be reported on reducing wet mycelial weights (p>0.05).

The effects of pH, ORP, and ACC on antifungal effects against *A. flavus* The pH, high ORP, and ACC of EOW caused a strong antifungal activity against *A flavus*. Growth of *A. flavus* mycelia was significantly inhibited by EOW (S1 and S3), compared to controls (p<0.01). The main factors that account for this antifungal activity against *A. flavus* were investigated. The effects of pH, ORP, and ACC on antifungal activity were evaluated. Surviving *A. flavus* conidial populations after treatment with a series of solutions (S1-S6) with differing pH, ORP, and ACC values were enumerated (Table 3, Fig. 2). The pH and ORP values of S1 were similar to S2, but the ACC values were different. The same relationship held for S3 and S4. The surviving fungal populations were significantly reduced

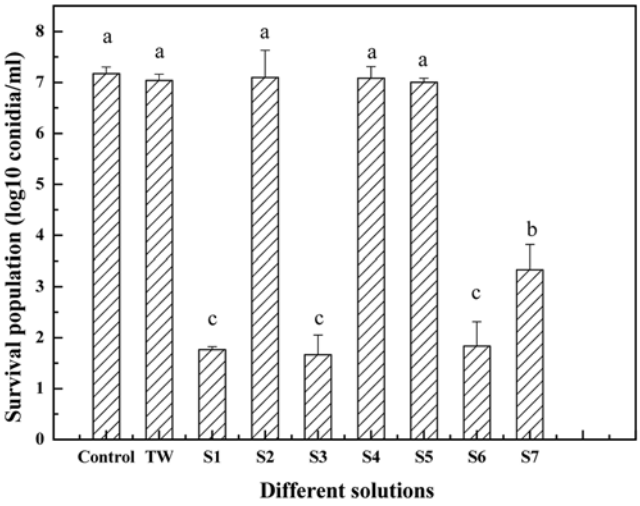

Fig. 1. Surviving population of *A.flavus* conidia after treatment with a series of solutions with different pH, ORP, and ACC values. TW, tap water

after treatments with solutions having similar pH and ORP values, but with different ACC values (S1 and S2) (p<0.01). However, surviving population levels after S2 and S4 treatments (lacking ACC) were not statistically different (p>0.05). The pH values of S3 and S6 were different, but the surviving population levels after S3 and S6 treatments were not significantly different (p>0.05). Solutions S2 and S5 had different ORP values; however, the surviving population levels after treatments showed no significant difference (p>0.05). These results indicate that ACC levels are an important factor in antifungal activities against *A. flavus*. Solutions S1, S3, and S6 overcame the resistance of *A. flavus* to available chlorine ionic compounds in a range of pH values from 2 to 6.

The antifungal strengths of S1 and S3 were stronger against *A. flavus* than S6. Although the ACC level of S7 was 10× more than the level of S6, the antifungal activity against *A. flavus* was not superior to S6 under alkaline conditions. Therefore, available chlorine ionic compounds

Table 3. Water properties and surviving populations of *A. flavus* conidia after electrolyzed water treatment[1)]

	EW type	Water properties			Treatment time (s)				
		pH	ORP (mV)	ACC (mg/L)	0	30	60	90	120
Surviving population (log_{10} conidia/mL)	AcEW	2.7±0.1[A2)]	1045±29[A]	28.0±1.1[A]	7.41±0.03[a3)]	6.08±0.06[b]	6.07±0.03[b]	6.00±0.03[bc]	5.90±0.02[c]
		2.5±0.1[A,B]	1097±28[A]	58.0±2.1[B]	7.41±0.03	ND[4)]	ND	ND	ND
		2.3±0.2[B]	1110±28[A]	86.1±2.2[C]	7.41±0.03	ND	ND	ND	ND
	NEW	6.2±0.3[A]	872±4[A]	29.2±0.7[A]	7.41±0.01[a]	5.39±0.13[b]	4.97±0.04[c]	ND	ND
		6.2±0.2[A]	870±21[A]	57.6±1.2[B]	7.41±0.01	ND	ND	ND	ND
		6.3±0.1[A]	867±11[A]	86.3±0.6[C]	7.41±0.01	ND	ND	ND	ND

[1)]Values are means of two replicated measurements±SD. Values with different superscripts are significantly different ($p<0.05$).
[2)A-C]indicating significant difference in the physicochemical properties (pH, ORP, and ACC) of water.
[3)a- c]indicating significant difference of surviving populations at different times ($p<0.05$)
[4)]ND, no detectable survivors using a direct plating procedure. Minimum level of detection was 10 CFU/mL of solution.

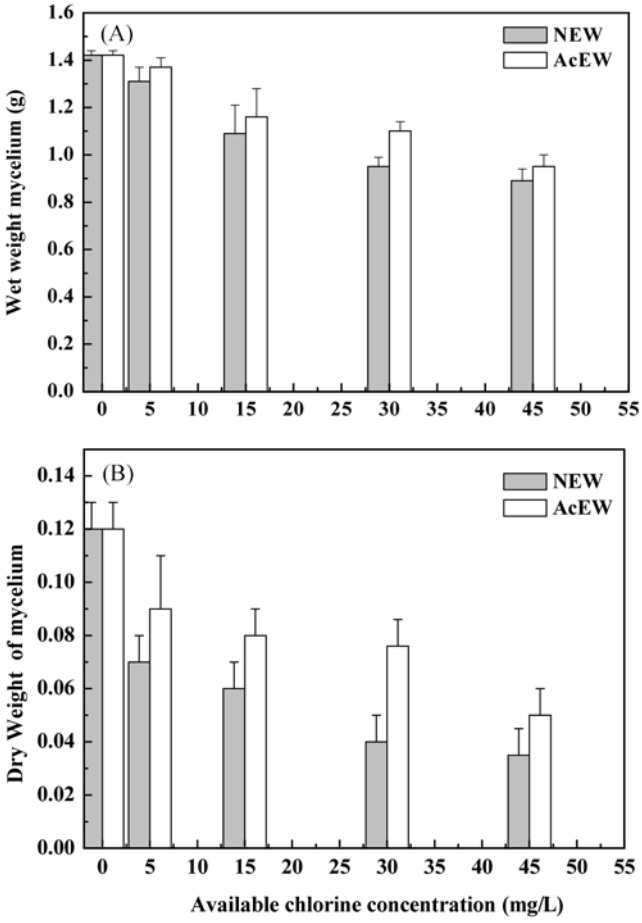

Fig. 2. Mean mycelial weight (g) of *A.flavus* treated with AcEW and NEW with different ACC values. (A), wet mycelial; (B), dry mycelial

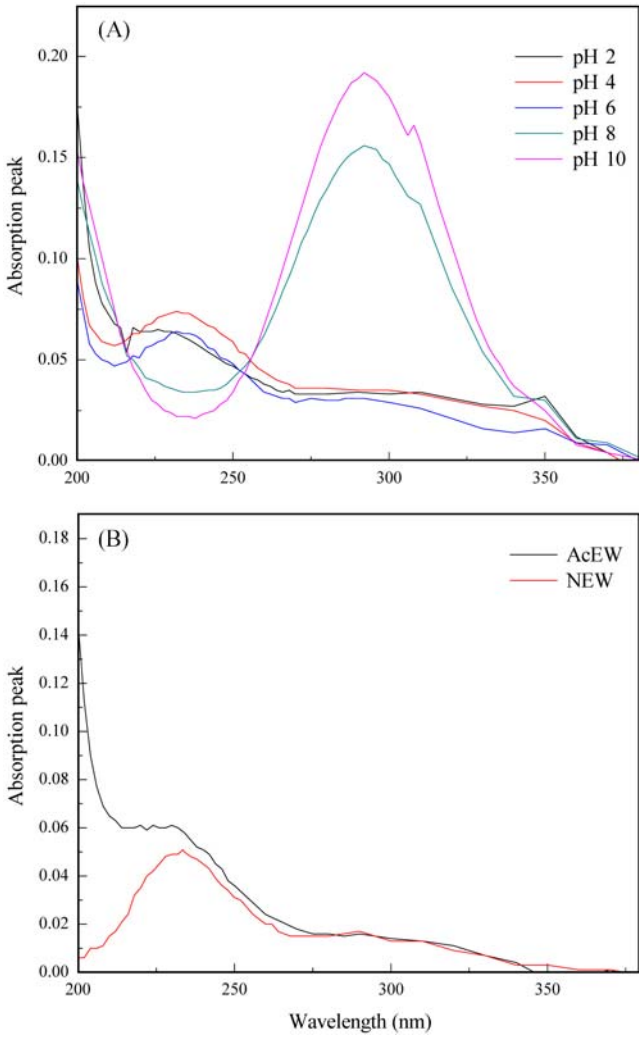

Fig. 3. Ultraviolet (UV) spectroscopy of the ClO^- in solution. (A), serial pH values of the NaClO solution; (B), in AcEW and NEW solutions

must be associated with the antifungal activity against *A. flavus* over a range of pH values (Fig. 3A). The dilute NaClO solution was analyzed from pH 2 to 10 at intervals of 2 using treatments with ultraviolet scanning in the range of 200 to 380 nm. The maximum absorption peak was measured at 292 nm, which is the characteristic absorption peak of ClO^- (27). Available chlorine usually exists in dilute NaClO solutions as ClO^- (28). Available chlorine ionic compounds form in the ClO^- form change to the HOCl form. The maximum absorption peak measured at the 292 nm was gradually reduced while the maximum

Table 4. The K^+ and Mg^{2+} permeability of conidia and mycelia treated with NEW and AcEW[1)] (unit: mg/L)

Group		K^+	Mg^{2+}
AcEW	Control	14.71±0.88	0.99±0.05
	Treatment	20.92±0.04	1.44±0.01
NEW	Control	15.47±0.07	5.70±0.02
	Treatment	18.75±0.01	6.28±0.06

[1)]Values are the means of two replicated measurements±SD.

absorption peak measured at the 232 nm (the characteristic absorption of HClO) was gradually increased when the pH value from 10 to 2. At pH 4 (approximately), the height of the maximum absorption peak at 232 nm was higher than at any other pH value. Characteristic absorption peaks of AcEW and NEW are shown in Fig. 3B. The maximum absorption peak measured at 232 nm coincided with the characteristic absorption peak of HClO. Therefore, ACC levels significantly affect the antifungal activity of EOW, in comparison with distill water and tap water (Table 3) ($p<0.01$). HOCl accounts for the available chlorine in the antifungal activity of EOW against *A. flavus*.

The K^+ and Mg^{2+} permeability of conidia treated with NEW and AcEW The K^+ and Mg^{2+} permeability of conidia are shown in Table 4. The permeability of K^+ and Mg^{2+} after treatment of *A. flavus* conidia and mycelia suspensions with NEW and AcEW were significantly increased ($p<0.05$), compared with the control. The leakage amount of K^+ was more the leakage amount of Mg^{2+}. NEW and AcEW damage the functional cellular structures of *A. flavus* conidia and mycelia to accelerate K^+ and Mg^{2+} permeability. Ion permeability reflects the normal state of conidia and mycelia. The ionic permeability is related to the selective absorption of conidia and mycelia. NEW and AcEW damage the conidia and mycelial structures, leading to changes in selective absorption. Conidia and mycelia finally lose normal function, causing leakage of K^+ and Mg^{2+} ions.

Scanning electron microscopy The morphology of the *A. flavus* mycelium is a raw stem with a regular shape. (Fig. 4A). Mycelia after treatment with AcEW observed under a scanning electron microscope are shown in Fig. 4B. Compared with the control (Fig. 4A), the mycelial wall is cracked with surface exfoliation and tearing of the fibrous structure with no regular shape. Conidia after treatment with NEW, observed under a scanning electron microscope, are shown in Fig. 4C. Mycelia are broken and deeply cracked and chipped. Compared with normal conidial (Fig. 4D), the morphology of conidia after treatment with AcEW become rough and the spiny sphere surface also become swollen, with node formation on the cell wall and the cell wall structure is deformed (Fig. 4E). The cell wall is cracked, shrunken, and leakage of cell contents is indicated by arrows. Conidia after treatment with NEW, observed under a scanning electron microscope, are shown in Fig. 4F. The cell wall is shrunken and partially cracked with holes, indicated by arrows.

Changes in morphological structures are connected with the conidium K^+ and Mg^{2+} permeability results showing that NEW and AcEW caused damage to conidia and mycelial cellular functional structures leading to accelerated ion leakage. However, these changes in morphology have been associated with conidia cell wall synthesis, cell membrane permeability, and conidium respiratory metabolism. Further research is needed to reveal what important factor in EOW destroys the normal conidia morphological structure.

This study has demonstrated that EOW has a strong antifungal activity against *A. flavus* conidia and inhibits growth of *A. flavus* mycelia (Table 2, Fig. 1). Available chlorine significantly affects antifungal activity against *A. flavus* compared with control ($p<0.01$). In addition to the superiority of EOW over tap water and distilled water to eliminate *A. flavus*, high ACC levels in EOW are beneficial for reductions of the surviving population and the mycelial weight of *A. flavus.* Park *et al.* (29) reported that the ACC level was more important for reducing conidial activity than the length of treatment. Thus, ACC should be considered as an important factor for evaluating the efficiency of EOW for inhibition and reduction of *A. flavus* contamination.

The EOW used in this study possessed unique physicochemical properties (pH, ORP, and ACC) (Table 1) those are probably important contributing factors to antifungal activities. Generally, *A. flavus* can flourish between pH 3 and 6, while aerobic fungi grow mostly at ORP values ranging from +200 to +800 mV. Compared with tap water and distilled water, NEW and AcEW exhibited higher ORP and lower pH values (Table 1) ($p<0.05$). Several theories for microbe inactivation have been proposed based on the ORP and pH values of EOW (27,30). Apparently, the *A. flavus* inactivation by AcEW and NEW can be attributed to the higher ORP and the lower pH values than for tap and distilled water. With low ACC levels in AcEW and NEW, the surviving population was not significantly reduced ($p>0.05$), compared with tap water and the control (S2 and S4 in Table 3). ORP and pH were not the main factors for control of antimicrobial activity because the higher ORP value of ozonated water and the lower pH of an HCl solution did not show a stronger disinfectant effect than lower ORP and higher pH values in EOW. This result does not support the theories for microbe inactivation based on the ORP and pH values. The chlorine molecule can adopt multiple forms, including

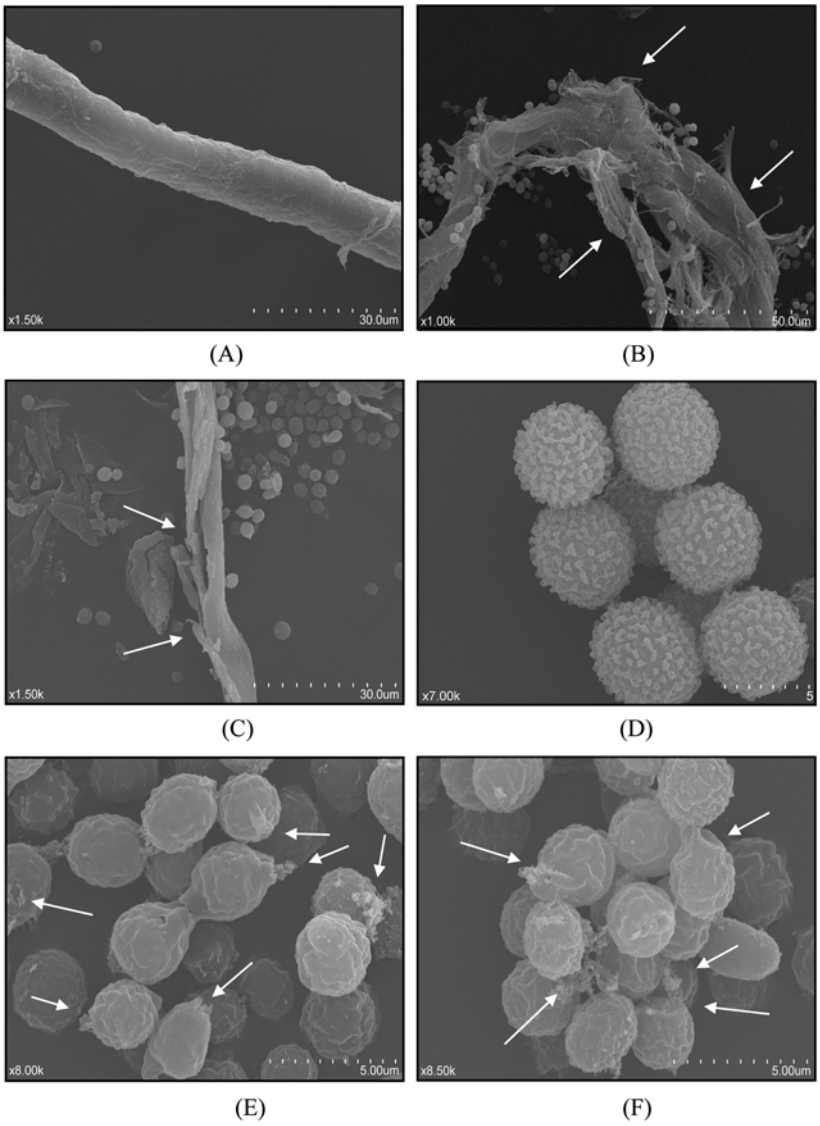

Fig. 4. Scanning electron photomicrographs of *A. flavus* conidia and mycelia. (A), the normal mycelium; (B), mycelia treated with AcEW; (C), mycelium treated with NEW; (D), the normal conidia; (E), conidia treated with AcEW; (F), conidia treated with NEW. Arrows show morphological changes in the conidia and mycelia, including cell wall shrinkage, partial cracking, chipping, and holes. Scale bars=(A and C), 30 μm; (B), 50 μm; (D-F), 5 μm

the ClO^- and HOCl forms (Fig. 3A). The characteristic absorption values AcEW and NEW coincided with a pH adjustment to the HOCl form. ClO^- and HOCl exist the dynamic balance reaction in solution as following:

$$HOCl\ H^+ + OCl^- \quad (1)$$

As pH reduced, reaction (1) reverses. The available chlorine compounds from the ion forms ClO^- change to the HOCl. Thus, HOCl is responsible for the antifungal effect against *A. flavus*. Furthermore, results indicate that the fungicidal efficiency of HOCl is greater than the efficiency of ClO^- (S6 and S7 in Table 3).

The HOCl that is present in NEW and AcEW is the most important factor in fungicidal activity against *A. flavus* and probably causes the damage noted previously to the normal morphological conidia and mycelial structures. Leakage of K^+ and Mg^{2+} also verified the presence of damage to the conidia and mycelial cells and membranes caused by HOCl. These morphological changes cause the conidia and mycelia to lose their normal functions.

Huang *et al.* (31) reported that the available chlorine of EOW, mainly as HOCl, can produce hydroxyl radicals (·OH) that can act on microorganisms. These radicals may increase the amount of damage to the normal morphological cell structures caused by AcEW and NEW. Cell damage may arise due to available chlorine, ·OH, or some other radicals. The ionic permeability of conidia and mycelia may cause a series of chain reactions extending into the

cell interior. However, further investigation is needed in order to interpret the mechanism of HOCl damage to the ionic permeability of *A. flavus* and whether the change hampers nutriment active transport in the plasma membrane, and influences nutrient selective absorption. NEW and AcEW can eliminate *A. flavus* mycelia on agricultural materials; however, elimination may require a longer time of EOW treatment, especially in the case of surface roughness or damaged material. Oomori *et al.* (32) documented bactericidal activity of EOW that caused deterioration of organic materials, including proteins and amino acids. Free available chlorine in EOW can react with organic materials and convert to the state of combine chlorine. The bactericidal activity produced by combine chlorine is lower than that of the free form of available chlorine (33).

A. flavus was eliminated by use of AcEW and NEW, a new alternative method of fungal control that is superior to some current physical methods and synthetic chemical fungicides. The change in morphological structure of conidia is due to the HOCl that exists in NEW and AcEW. These changes are associated with conidia cell wall synthesis, cell membrane permeability, and conidia respiratory metabolism. Whether these changes cause effects on conidia development, aflatoxin production, or regulation of aflatoxin biosynthesis requires further research.

Acknowledgments This work was supported by a grant from the Beijing Natural Science Foundation, The mechanism of Ochratoxin A(OTA)-detoxifying enzyme and safety evaluation of its produces (supported No.5144026) and National Science-Technology Support Plan, Research and Demonstration on Key Technology and Equipment and Materials of Food Packing (2011BAD24B01).

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