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Effectiveness of Ozone Gas in Raw and Processed Food for Fungi and Mycotoxin Decontamination - A Review

Divair Christ [1,2] Geovana D. Savi [1], Vildes M. Scussel [1]

[1]Laboratory of Mycotoxicology and Food Contaminants, LABMICO, Food Science and Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, Rod. Admar Gonzaga, Itacorubi, 1346, Florianopolis, SC, Brazil. www.labmico.ufsc.br.

[2]Storage Laboratory and Prototypes Drying Facilities, PGEAGRI, Technological and Exact Sciences Center, Western Parana State University, R. Universitaria, 1619, Jardim Universitario, Cascavel, PR, Brazil.

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Abstract: This review gathers information on ozone (O_3), a Food and Agricultural Organization, US Agriculture Department & Food and Drugs Administration generally recognized as safe (GRAS) gas for use in food processing (decomposition to molecular oxygen without leaving residue). It brings details on the energy source for O_3 formation, application characteristics and decontamination effect in different food, focusing on fungi inactivation and mycotoxins degradation. A comparison on literature methodology of application regarding O_3 *gas* (concentration and time of exposure); *food* (type, contamination level or batch size) and *container* (volume, material type, sealed or hermetic), including the effect on *fungi* (total load, genera, species and spores susceptibility), their *toxins* (aflatoxins, deoxynivalenol, fumonisins, citrinin, patulin) and its *efficiency*/*viability* for fresh, stored raw and processed foods, are covered. From the studies and data reported, O_3 has shown to be a promising and efficient decontamination "green" agent for fungi and their toxins in food (low or high humidity), prolonging the storage and shelf life time.

Keywords: ozone, decontamination, mycotoxin, fungi, food safety.

INTRODUCTION

Background

The increasing concern on environmental safety and human health, has stimulated the development and/or improvement of non-aggressive food disinfection/decontamination oxidizing substances in order to avoid and/or minimize their application impact [1-6]. An oxidant, acceptable from the environmental/health point of view must have the following characteristics: to (a) *react specifically* with the living organism/compound to be destroyed/degraded; (b) *not form toxic by-products* (with toxicity equal to or higher than the target contaminant) and (c) *be easy to obtain*[7,8].

Decontaminant agents

Different oxidizing agents have been reported and applied as decontaminants to destroy living organisms (bacteria, fungi, yeast, viruses, protozoa, insects and mites) and/or degrade toxic compounds (pesticides, mycotoxins and industry toxic wastes). The chemical compounds most commonly used are: hydrogen peroxide, chlorine, chlorine dioxide, sodium hypochlorite and potassium permanganate[9-14]. However, their major application concerns are the residues left in food and consumer safety. The "green" method that has been shown its decontamination efficiency to post-harvest high (fruits and vegetables) and low (grain, nuts and pulses) humidity food, without leaving residue is ozone (O_3), either as gas or in the liquid form [15-19].

OZONE GAS

O_3, the triatomic allotrope of O_2, is formed when O_2 molecules are broken into individual oxygen atoms, which combine with other O_2[20]. Due to O_2 high stability, O_3 molecule undergoes a spontaneous dissociation process, again resulting in O_2 formation [21]. The O_3 bluish gas decomposition is characterized by a rapid decrease of the initial concentration, followed by a second phase in which O_3 concentration decreases as first-order kinetics. Its half life time varies from a few seconds to hours, and stability depends on factors, such as pH (aqueous O_3) and temperature (gaseousO_3), where a 10ºC increase, results in 43% half-life reduction [22]. The O_3 half-life in atmospheric conditions is about 30 min and reduces with higher temperatures and low pressures [23, 24].

Therefore, being an unstable gas, it requires to be produced at its application site, reducing costs and risks related to transportation and storage [1, 9]. As a very reactive oxidizing agent, O_3 has proved effective against a broad spectrum of living organisms and chemicals, including bacteria [17, 23, 24], fungi [25, 26], yeasts [27-29], viruses [21, 30, 31] and protozoa [32]. It also has the potential to kill storage pests, such as insects [16]& mites [33], as well as, degrade mycotoxins [31], pesticides [34, 35] and toxic chemical wastes [9, 22, 36, 37].

Ozone energy sources and apparatus

The O_3 formation can occur both (a) naturally and freely in the stratosphere through the interaction of solar ultraviolet radiation with the molecular O_2 and (b) artificially through electric dischargers reaction or ionizing radiation, being the corona discharger apparatus, the most known and utilized in different food processes [20, 38].

There are different types, sizes and O_3 generation capacity ozonators, from laboratory (small sizes and low capacities) to storage unities (large sizes and high capacity) for a broad range of applications. Some of them can be small (portable for laboratory use), medium (installed along the process plant in industries) or large (for grain storage unities) sizes. Their capacities can vary from 125g/h up to 10 kg/h with electronic controls and alarms[39, 40].

Ozone generally recognized as safe applications

As O_3 gas spontaneously converts it molecules into O_2 and does not result in harmful species, it has been considered by different international organizations and countries regulations as GRAS (generally recognized as safe) to be utilized in direct contact to drinking water and food (the US Food and Drug Administration – FDA& Agriculture Department - USDA, Food Agriculture Organization - FAO and World Health Organization - WHO). FDA/USDA: since 1975 FDA recognizes the O_3 treatment a GMP (Good Manufacturing Practice) method for the bottled water industry and in 1997,recognized it as GRAS for use in food processing [41, 42]. Further on, O_3 was accepted for legal use directly in food processing and agricultural products as antimicrobial agent [43], including meat and poultry (both in gas & aqueous phases). That also was for raw materials and minimally processed vegetables & fruits [7, 41, 42, 44].

FAO/WHO: approved O_3 for bacterial water control as well as food quality and safety controls in the processing systems since 1983 [45, 46]. It also defines the benefits and risks of the O_3 use as disinfectant in the food production are details [13]. Countries from different continents also recognize the O_3 application mainly in water followed by food. Europe – the European Commission (EC): directive 2003/40 [47] established, apart from the list type, concentration limits and labeling requirements for the constituents of drinking water (natural mineral and spring), it includes the water O_3-enriched and set the O_3 residue maximum limit (50 µg/l) in natural mineral and spring waters O_3-enriched [47].

Brazil: the Brazilian Agricultural and Health Ministries approved the O_3 application in equipment for filling, closing (including the utensils that come in contact to) water [48]. It also established in 1999 standard regulation for purified water, in which bottles labels should inform the purification treatment applied, inclusive O_3[49]. The Agriculture Ministry [50] established an O_3 regulation for pesticide residues decontamination(organophosphates) in airplanes applicators parking area (to date, worldwide, such waste is not treated; or it is just dumped - contaminating rivers, lakes and groundwater. Japanese, Australian and Chinese regulations: the O_3 use in food has been allowed, either in the factory air treatment, the water and food products, as well food materials and food processing plants [51-53]. Apart from those international institutions and countries Agriculture and Health Ministries recognizing the use of O_3 for food, there is a wide supporting literature attesting the benefits of ozonation as an efficient method/procedure for food (raw & processed) living organisms inactivation and chemicals degradation, some of them will be reviewed herein.

OZONE GAS APPLICATIONS IN THE FOOD AREA

Living organisms inactivation: $O_3$is reported in food against bacteria (*Salmonella; Escherichia; Pseudomonas; Staphylococcus*)[17, 24, 29, 30, 54], fungi (*Botrytis, Fusarium, Aspergillus, Penicillium*)[2-5, 16,55-64], yeasts[27, 28], insects (*Sitophilus, Rhyzopertha, Tribolium*)[16, 33, 39, 65-70], mites (*Tyrophagus; Dermatophagoides*)[71] and protozoa (*Giardia, Endamoeba, Leichmania*)[32, 72-78]. Those living organisms proliferation may contaminate food prior and after processing affecting their sanitary conditions to consumers. They may cause either: to food - deterioration (bacteria, fungi, insects) and/or toxins transfer, thus reducing quality and safety; and to humans - develop of diseases or infections by

bacteria, parasites and protozoa by food ingestion or intoxication symptoms (bacteria) and allergies (mites).

Toxic chemical contaminants degradation: Regarding different food chemical contaminants degraded by O_3, there are the pesticides [6, 34, 35, 52, 79-82], mycotoxins [4, 5, 53, 55, 63, 83-87] and industry toxic wastes [6, 9, 22, 36, 37, 80, 82]. Their degradation occurs because O_3 is able to participate in a large number of reactions, mainly with those compounds that contain double bonds such as C=C, C=N, N=N [22, 36].

OZONE GAS EFFICIENCY AGAINST FUNGI AND MYCOTOXINS IN FOOD:

Data on O_3 food treatments - for fungi (spoilage and toxigenic) inhibition and mycotoxins (field and storage) degradation - has been published in less extent, when compared to the focus on bacteria. However to date, there have been enough data to conclude that O_3 is efficient for their decontamination. **Tables-1** and **2** present food studies carried out against both contaminants reported in the literature, respectively.

They show treatment differences such as: whether applied on naturally contaminated[53, 55, 88-90] or inoculated/spiked on food samples [4, 5, 39, 90-92]. The gas concentrations; exposure time *versus* food contamination levels and the food group (cereals, pulses, nuts and fruits) are also detailed including the percentage of contaminant reduction. Some of them do inform their effectiveness related to the initial (fungi load and toxin level - whether high or just above the MTL allowed) and final (after gas treatment) food contamination level, which are quite important information to achieve the O_3 method concentration effectiveness. Also, some of them do inform fungi genera & species differences on susceptibility to that gas and evaluate a broader range of toxins from field (deoxynivalenol – DON, fumonisins – FBs) & storage (aflatoxins – AFL, citrinin – CTL, patulin – PTL) fungi origin, that have MTL set by different countries regulations, including Brazil and Mercosur [41, 42, 47, 93].

FUNGI INACTIVATION

In post-harvest, the food (raw, dry or processed) O_3 treatments have been carried out aiming to improve quality and prevent quantitative losses due to fungi spoilage, either in cereals (maize, wheat, rice, barley), pulses (peanuts, peas, lentils), nuts (pistachio, Brazil nuts), fruits (fresh: grapes, kiwi, lemons, oranges and dry: figs, raisins) and cheese. Details on antifungal O_3 treatments by different authors for different food are shown in **Table-1**. Most of them report their effects on fungi load (total fungi count-TFC), aiming only to reduce/inactivate any fungi colony or spores load present on/in the food [2, 4, 5, 27, 28, 39, 57, 64, 85, 91, 94-97]. On the other hand, several of them specify fungi genera characteristics, whether toxigenic or only deteriorating strains. Only a few goes into details checking O_3 gas concentrations and time variation among different fungi genera and species for their specific susceptibility (which is shown to vary with the different conditions applied) in order to achieve the best effectiveness [3-5, 16, 39, 56, 62-64, 91, 92, 98, 99]. Those information are important on deciding the efficacy of the method to be applied in commercial processes. The fungi contamination extent estimation on the food to be treated, prior O_3 gas application, will also help to achieve the best performance.

Table-1: Ozone applications for fungi inhibition in different foods reported in the literature.

Food	O_3 treatment					Storage (days)	Fungi & yeasts				Culture media	Reference
	Concentration	Unity*	Time (min)	Flow	Unity*		TFC(CFU/g) Initial	TFC(CFU/g) After O_3	Inhibition (%)	Genera & species (isolated/identified/studied)		
Cereals												
Barley	0.16/0.1	mg/g x min	5.0	NI	NI	NI	8.3×10^5	NI	96.0	NI	PDA[a]	[94]
	11/26	mg/g	15/30/60	NI	NI	NI	NI	NI	24.0/36.0	*Fusarium*	NI	[56]
	3	%	1.0/1.5/3.0	NI	NI	NA	NI	NI	100	*Penicillium; Aspergillus; Fusarium; Alternaria; Rhizoctonia*; *Acremonium*; *Mucor*	PDA	[99]
Maize	5	ppm	5 days	NI	NI	NA	NI	NI	100	*A. flavus; F. verticillioides*	NI	[98]
	20/50	ppm	3days	0.054/0.019	m^3/s	NA	43×10^5	16×10^5	63.0	*A. parasiticus*	PDA	[16]
	47.800	ppm	1.8[b]	258.120	ppm-min	NA	10.6×10^3	68[c]	99.3/99.9	*A.flavus*	MSM[d]	[39]
	50/500/1000/15000[e]	ppm	60.0	0.5	l/min	NA	NI	NI[f]	58.0/76.0/50.0/73.0[g]	*Aspergillus, Fusarium, Mucor; Penicillium*	MEA[h]	[62]
	50/100/200	ppm	60.0	6.0	l/min	15/30	2.2[i]	ND[j]	100	*F. verticillioides*	MMAM[k]	[63]
Wheat	280/700	ppb	64h	0.12	m/s	NA	NI	NI	100[l]	*A. alternata, F. avenaceum, F. graminearum*	MCME[m]	[100]
	NI	NI	5.0	0.33	mg/g/min	NA	4.9×10^5	NI	96.9	Fungi (NI)	PDA	[57]
	3	%	1.0/1.5/3.0	NI	NI	NA	NI	NI	100[n]	*Penicillium; Aspergillus; Fusarium; Alternaria; Rhizoctonia; Acremonium*; *Mucor;*	PDA	[99]
	20/40	ppm	5/10/15/20	NI	NI	NI	1×10^5	NI	95.6	*A. flavus*	PDA	[91]
	20/40	ppm	5/10/15/20	NI	NI	90	1×10^2	NI	NI[o]	*A. flavus*	PDA	[92]
	40/60	µmoL/mol	30/60/120/180	1.0	l/min	NA	48x10	ND	100[p]	*F. graminearum*	PDA	[4]
	40/60	µmoL/mol	30/60/120/180	1.0	l/min	NA	44×10	5.35×10^1	87.8	*A. flavus*; *A. parasiticus; P. citrinum; F. verticilioides;* *A. flavus*; *P. citrinum*	PDA	[3] [5]
Rice[q]	10/20/40	mg/l	30.0	1.0	l/min	NA	3×10^5	1.4×10^2	99.9	*Aspergillus*; *Penicillium; Acremonium; Alternaria*	PDA	[64]
Pulses &Nuts												
Peanuts	13/21	mg/l	24/48/72/96h	1.0	l/min	NA	6 log	3 log	3 log	*A. flavus; A. parasiticus*	PDA	[96]
Peas	3	%	1.0/1.5/3.0	NI	NI	NA	NI	NI	100[t]	*Penicillium; Aspergillus; Fusarium; Alternaria; Rhizoctonia; Acremonium; Mucor*	PDA	[99]
Brazil nuts	10	mg/l	90.0	NI		1/30/60	1.83×10^4	ND	100	*A. flavus*; *A. parasiticus*	agar	[85]
	10/14/31.5	mg/l	180/300	NI		180	4.83 log	ND	100	*A. flavus*; *A. parasiticus*	MEA	[2]
Fruits												
Citrus	0.3/1.0	ppm	continuous	NA	NI	14	10^6	NA	5	*P. italicum /P. digitatum*	PDA	[26]
Dates	1/3/5	ppm	15/30/45/60	NI	NI	NI	3.93	3.61	NI	Yeast/fungi	YGC[r]	[28]
Figs	1/5/10	ppm	180/300	5.0	g/h	NI	1.46 log	0.40 log	72.0	Yeast/fungi	PDA	[27]
	13.8	mg/l	7.5/15/30	6.0	l/min	NI	1.73 log	ND	100	*A. flavus; A. niger; A. parasiticus; Cladosporium hiemalis Byssochlamys; Mucor; Scopulariopsis*	PDA	[95]
Grapes	200[s]	ppm	2/12/12 h	800/1200/2000	ppm/h	7	NI	NI	99.0	*P. digitatum; P. italicum; B. cinerea*	PDA	[61]
	75/100/150/200/250/300/500 0	ppm	NI	NI	NI	NI	10^6 spores m/l	NI	65.0	*B. cinerea*	NI	[97]

* unities as referred by the authors [a]potato dextrose agar [b]3x[c]dry ozone [d]malt salt medium (2% malt extract, 6%NaCl, and 1.5% agar) [e]high humidity (18/22/26%) [t]colonies observed per 100 kernels[g]*Aspergillus, Fusarium &Mucor*treated at 500 ppm and more *Penicillium* more resistant at 15,000 ppm [h]malt extract agar [i]at0.88 *aw*[j]not detected [k]milled maize agar media [k]same Micomycetes stopped active functioning [m]malt, czapek and maize extract [n]*Penicillium* spp inhibition was 95% [o]inhibit production of AFB_1 by *A. flavus*[p]180 min[q]paddy rice [r]yeast extract glucose chloramphenicol agar [s]35, 75and 95% RH [t]*Aspergillus* spp inhibition was 92%. NI: no indicated; NA: not applied.

Table-2: Ozone applications for aflatoxins and others mycotoxins degradation in different foods reported in the literature.

Food					O_3 treatment				AFLs						Method applied		
	Quantity	AFLs initial (µg/kg)		Silo load (l)			Time	Storage	Degradation (µg/kg)					Inhibition			Reference
Type	(kg)	Artificial	Natural	Bulk	Conc	Unity[a]	(min)	(days)	AFB_1	AFB_2	AFG_1	AFG_2	AFL_{total}	(%)	Detection	LOD[b]&LOQ[c]	
AFLATOXINS																	
Cereals																	
Maize	NI	NI	NI	NI	5	ppm	5 days	NA	NI	NI	NI	NI	NI	99	NI	NI	[98]
	0.001	NI	NA	NI	20	%wt	5	NA	NI	NI	NI	NI	NI	66.9/59.8 80.8/23.5[a]	LC	NI	[109][e]
	30	NA	1,220[t]	NI	200	mg/min	5,520 (92h)	NI	58.4	NA	NA	NA	NA	95.0	LC	NI	[55]
	10	586,8	<2	NA	12	%wt	96 h	NA	47.7 / <2	NI	NI	NI	NI	92	LC	NI	[110]
	220 kg/h	17.6	NA	NI	47,800	ppm	1.8 (3x)[g]	NI	NI	NI	NI	NI	5.7	20.0-30.0	TLC[h]	NI	[39]
	0.1	NA[t]	83.0[j]	1	40/65/90	mg/l	5 - 40	NI[k]	9.9	NA	NA	NA	NA	88.1	LC	NI	[53]
Wheat	NI	10.0/20.0[t]	NA	NI	20/40	ppm	5 - 20	NI	0.66	NA	NA	NA	NA	96.7	LC	NI	[91]
	0.35	231.9 /265.8 239.9 / 199.4	NA	2	40/60	µmol/mol	30 - 180	NA	12.51	41.06	47.96	37.81	42.90 (CTR)	94.6 / 84.5 80.0/ 81.0	LC/FLD	0.26 & 3.1 / 0.002 & 0.02 / 0.28 & 1.41 / 0.005 & 0.03	[5]
Pulses &nuts																	
Peanuts	NI	NA	82	NI	0.025	g/min	60	NA	18 ppb	NI	NI	NI	NI	78.0	TLC	NI	[88]
	0.025	20 ng/g	NA	NI	4.2	%/weight/ 15 psi	5 - 15 25/50/75 °C	NI	NI	NI	NI	NI	NI	77 / 51 80 /51	LC	NI	[83]
	1	NA	190	3	13/21	mg/l	24 - 96h	NI	134	NI	NI	NI	138	25	LC	NI	[96]
	NI	87.5/22.0/9.7/4. 4	NA	NI	89	mg/l	30	NI	15.23	8.31	2.81	2.11	NI	82.6 / 62.2 71.1 / 51.8	NI	NI	[111]
	NI	189.5/[m]105.35[n]	6.36 / 4.94	NI	50	mg/l	3600/7200[o]	NI	2.83/14.61.3 2 /5.79	NI	NI	NI	NI	55.5 / 92.3[p]/73.3 / 94.5	LC	NI	[90]
	1	NA	200	20	3.0/4.5/6.0/7.5	mg/l	10 - 120	NA	NI	NI	NI	NI	NI	62.1 / 43.0 / 78.0 / 64.0	LC	NI	[86]
Brazil nut	10	NA	10.6	0.26	10	mg/l	90	1.0 - 60	NI	NI	NI	NI	<0.36	100	LC-MS[n]	NI / 0.36	[85]
	2	NA	3.5/1.2 3.6/1. 9 2.2/2.0 2.3/1.7 11.6/6.0[q]	14.1	10/14/31.5	mg/l	60 - 300	1.0 - 180	ND	ND	ND	ND	ND	100	LC/FLD	NI & 0.5 / NI & 0.17 / NI & 0.5 / NI & 0.17 /NI & 1.34 AFG_{total}	[2]
Others																	
Chili[r]	0.075	NA	20.0/32.0[s]	NI	16/33/66	mg/l	7.5 - 60	NI	4/2	NI	NI	NI	NI	80/93	TLC	NI	[89]
Figs	0.2	21.0	NA	3	13.8/1.71[t]	mg/l	30 - 180	NI	1.01/2.39	NI	NI	NI	NI	95.2	LC	NI	[95]
OTHER TOXINS																	
Apple juice	0.03	$2.4 \cdot 10^{-3}$ [u]	PTL	NA	12	%	NI	NI	NA	NA	NA	NA	ND	100	LC-DAD	NI	[116]
Barley			DON		26	mg/cm³								inconclusive			[113]
Maize	0.075	NA	FBs[v]	NA	0/100/200	ppm	60	15/30	NA	NA	NA	NA	ND	100[z]	LC-MS[n]	NI	[63]
Wheat	0.35	1065.10	DON[*]	2	40/60	µmol/mol	30 - 180	NA	NA	NA	NA	NA	NA	100	LC/UV	67 / 119	[4]
	0.35	173.5[b]	CTR[#]	2	40/60	µmol/mol	30 -180	NA	12.51	41.06	47.96	37.81	42.90	75.3	LC/FLD	0.2 & 1.2 CTR	[5]

[a]unities as referred by the authors [b]limit of detection [c]limit of quantification [d]AFB_1; AFB_2; AFG_1; AFG_2 , respectively [e]maize powder [f]ppb, AFB_1 [g]258.120 ppm/min (In a modified screw conveyor) [h]thin layer chromatography [i]not applicable [j]mg/kg [k]not informed [l]AFB_1 [m]grains [n]peanut pastes [o]5 l / min [p]natural/artificial contamination [q]AFB_1 AFB_2 AFG_1 AFG_2 AFL_{total} in-shell/after shelling [r]red pepper [s]flaked/chopped [t]O_3 gas/ozonated [u]mM of patulin [v]fumonisisns: FB_1, FB_2, FB_3 [z]Not detected fumonisins: FB_1, FB_2, FB_3 at 200 ppm treatment O_3 [*]deoxynivalenol [#]citrin

(a) Effect against fungi in foods

(a.1) Cereals: most studies focus on reducing the TFC and were carried out mainly for maize, wheat, rice and barley. The concentration of O_3 gas to be applied vary quite widely from, as low as 0.1 to as high as 48,800 ppm (in a very short time - 1.8 min, though) (**Table-1**). Low concentrations of O_3 (5 ppm) for a long period of exposure (5 days) inhibited 100% of toxigenic *A. flavus* and *F. verticillioides* in maize [98]. Kells and others [16] studied the application of 50 ppm of $O_3$for 3 daysin that grain and found 63% inhibition of toxigenic *A. parasiticus*. McDonough and others [39] evaluated the use of a quite high O_3 concentrations (48,000 ppm) for a short exposure time (1.8 min) in a maize screw conveyor and reported a *A. flavus* reduction from $10.6x10^3$ to $6.8x10^1$ CFU/g (corresponding to 99.9% of contaminating strain).

White and others [62] evaluated also the O_3 application in maize (with high moisture content - mc) at concentration of up to 15,000 ppm for 60 min and reported reductions of 58.0, 76.0, 50.0 and 73.0% for *Aspergillus*, *Fusarium*, *Mucor* and *Penicillium*, respectively. Recently, studies conducted by Mylona and others [63] reported 100% reduction in maize contaminated with *F. verticillioides* by applying 200 ppm O_3 during 60 min. Raila and others [100] applied high concentration of O_3 (700 ppm) for 63 hours in wheat and removed 100% of contaminating of *Alternaria alternata, F. avenaceum*and *F. graminearum.*

Application of 0.33 mg/g/min during 5 min in wheat with $4.9x10^5$ CFU/g fungi load promoted 96.9%reduction[57]. Ciccarese and others [99] applied air at concentration with 3% O_3 for 3 min,observing 100% inhibition of *Aspergillus* spp., *Fusarium* spp., *Alternaria, Rhizoctonia, Acremonium,Mucor* and 95% of *Penicillium.* El-Desouky and others [91] studied the application of O_3in wheat atconcentration of 40 ppm during 20 min and observed reduction of 95.6% of *A. flavus*. On the other hand, the maize at mc of 18, 22 and 26% treated with O_3 air at high concentrations (500 and 1000 ppm) for 1 h were more effective on reducing *Aspergillus*, *Fusarium* and *Mucor* than *Penicillium* and *Rhizopus*. Indeed, *Penicillium* infections in maize seem to be more resistant and need longer exposure. It decreases with O_3 concentrations of 1,000 and 15,000 ppm, being a higher O_3 concentration (15,000 ppm) necessary to reduce *Rhizopus* infection [62]. When fungi inoculated in wheat were treated with O_3, *Aspergillus* and *Penicillium* showed to be more resistant than *Fusarium* at concentrations of 40 and 60 ppm and different times (30, 60, 120 and 180min) of exposure [4, 5].

In study of Savi and others [4], *F. graminearum* growth was significantly reduced ($12.5x10^1$ and $4x10^1$ CFU/g) in the O_3 Treated Group after 30 min of exposure at concentrations of 40 and 60 µmol/mol when compared to the Control Group (not Treated), that represents a rate of 74.5 and 91.8% spores inhibition. In turn, after 180 min of O_3 exposition (at the same concentrations) *F. graminearum* growth was totally inhibited. In addition, the same authors, showed that *A. flavus* growth was significantly reduced ($8.5x10^1$ and $5.35x10^1$ CFU/g) after 30 min of O_3 exposure at concentrations of 40 and 60µmol/mol, respectively (when compared to the Control Group that represents 80.7 and 87.8% spores inhibition). The total *A. flavus* growth inhibition was only registered at the highest O_3 exposure of (60 µmol/mol) after 180 min. *P. citrinum* also was significantly reduced ($8.4x10^1$ and $6.9x10^1$ CFU/g) after 30 min of O_3 exposure (both at 40 and 60 µmol/mol) with 67.6 and 73.4% spores inhibition. That fungi strain total growth inhibition occurred after O_3treatment with 60 µmol/mol during 180 min of exposure [5]. Also wheat grains had their initial fungi load of 4.9×10^5 CFU/g reduced in 96.9% at gas application of 0.33 mg/g/min during 5 min [57].

When paddy rice was O_3 treated (40 mg/l; 30 min), the fungi growth reduction (*Aspergillus, Penicillium, Acremonium, Alternaria* and *Aureobasidium*) was of 99.9% (from $3x10^5$to

$1.4x10^2$CFU/g). Data suggest an O_3 certain resistance by the genera *Aureobasidium*, *Aspergillus* and *Penicillium* as well as for yeasts to the conditions applied. The occurrence of fungi and yeasts simultaneously and only yeasts for each O_3 treatment was significantly different (P<0.05), confirming that yeasts are more resistant than fungi in the study conditions [64].

(a.2) Pulses, nuts and dries fruits: although soybean is the main pulse produced worldwide, no study has been carried out on O_3 to date, to our knowledge. However, peanuts [96] and pea [99] have been studied with quite good results of fungi reduction and spore inactivation (100%) including *A. flavus*, *A. parasiticus, Penicillium, Fusarium, Alternaria*, *Rhizoctonia, Acremonium* and *Mucor*. TheBrazil nuts [2, 85] when O_3 treated at 10 to 31.5 mg/l, had also 100% reduction for *A. flavus* and *A.parasiticus*. Regarding dry fruits fungi control, their raw material (fresh fruits), which also are most prone to fungi and toxins contamination, especially when the ones utilized for dehydration are of low quality (i.e., already fungi deteriorated) - they can lead and end up to similar (or higher) contamination final dry product. Several studies for dates, figs, citrus and grapes have reported O_3 gas efficiency in the literature [89, 95, 101].

(b) Mechanisms of living organism's inactivation

(b.1) Cells membrane, spore coats and germination effect: the O_3microorganism's inactivation occurs in a complex process as that gas attacks several cellular chemical constituents of different *cellmembranes* (proteins, unsaturated lipids and respiratory enzymes), *cytoplasm* (enzymes and nucleicacids) and *spore coats* (proteins and peptidoglycan) [21, 102, 103].Although some authors conclude that the molecular O_3 is the main microorganism's inactivator, others emphasize the O_3 decomposition reactive by-products, as the antimicrobial activity responsible [104-108]. Indeed, O_3 oxidizes several compounds responsible for the cell membrane structure integrity leading to contents leakage and cell lysis[102,103].

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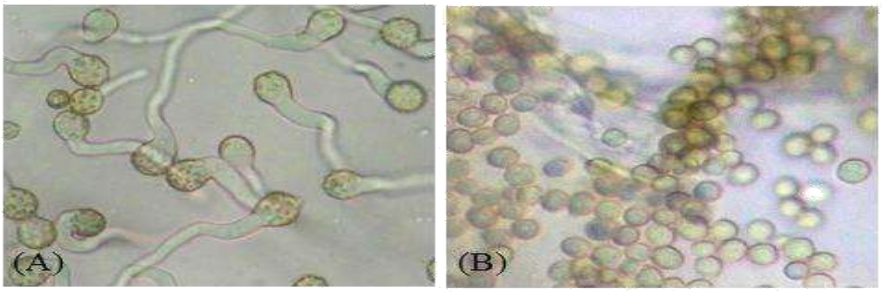

Figure 1: Ozone gas effect on fungi germination: *Aspergillus* spores [A] before and [B] after ozone treatment [3].

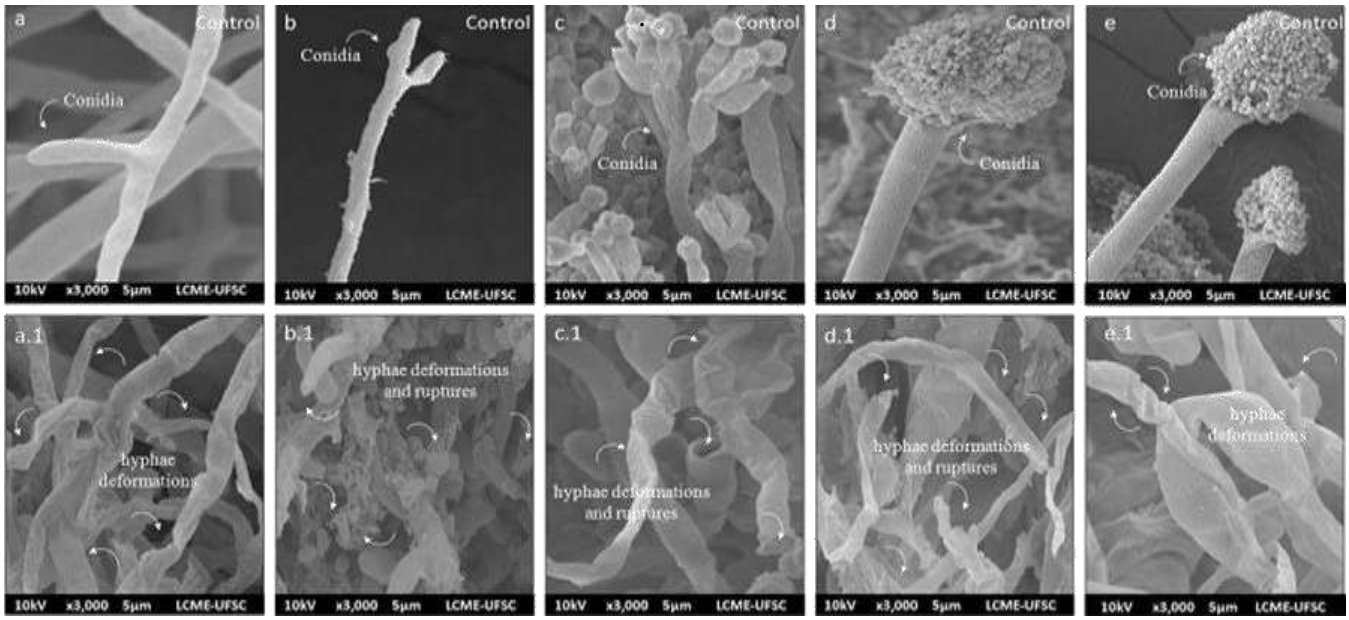

Figure 2 Ozone gas effect (60 ppm, 90 min) on hyphae morphology of (a.1) *Fusarium graminearum*, (b) *F. verticillioides*, (c) *Penicillium citrinum*, (d) *Aspergillus parasiticus* and (e) *A. flavus*[3].

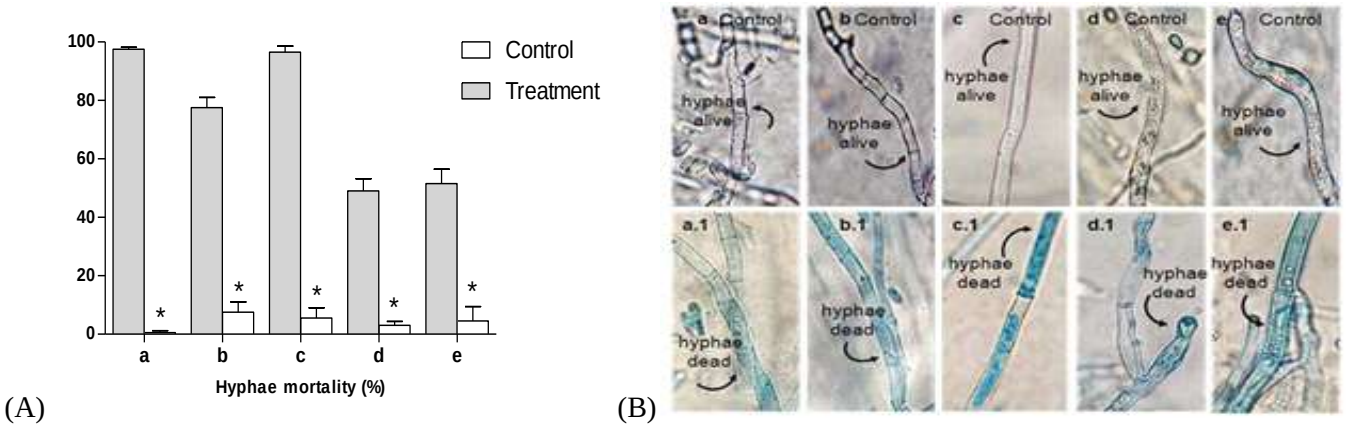

Figure 3: Ozone gas effect on hyphae mortality (A) as percentage (average and standard deviation) and (B) by Evans blue staining distribution in (a) *Fusarium graminearum*, (b) *F. verticillioides*, (c) *P. citrinum*, (d) *Aspergillus parasiticus* and (e) *A. flavus* Control and [1]Treated[3]

Disruption of membrane and spore coat: when the unsaturated lipids (double bonds) and the enzymes (sulfhydryl groups) are O_3 oxidized, occurs *disruption* of the regular cellular activities, causing alteration of the cell permeability and *rapid death* takes place. *Inhibition of spores germination:* similarly the membrane, spore coats also suffers the O_3 effects. In a work carried out by Savi and others [4], fungi spores affected by O_3 gas lost the ability to germinate. Probably, the coat cell membranes alterations occurred, similar to those of bacteria, keeping the respective characteristics of robustness (fungi spores are much more resistant). The authors showed that conidia germination was strongly inhibited by the O_3 gas treatment when compared with the Control group. The most effective conidia germination inhibition was observed at the longest period of O_3 gas exposure (120 min at 60 ppm). Under this treatment, the reduction of *F. verticillioides*, *A. parasiticus*, *A. flavus*, *P. citrinum* and *F. graminearum conidia* germination were 39, 27, 17, 9 and 3%, respectively, when compared to Control (80, 96, 84, 98 and 99%, respectively). **Figure 1** shows the effect of O_3 gas treatment on *A. flavus* spore germination.

(b.2) Hyphae morphology, mortality and ROS effects: The effect of the O_3 gas direct exposure on fungi morphological alterations and cell death still is not understood. Therefore, knowing the mechanism of action of the O_3 gas exposure on filamentous fungi is essential to evaluated its efficacy as decontamination agent. Hyphae morphology: in a study to evaluate the gas effect (at 60 ppm and 90 min exposure) on fungi (*F. graminearum, F. verticillioides, P. citrinum, A. parasiticus* and *A. flavus*) strains hyphae morphology and growth development, Savi and Scussel [3] showed that the O_3 gas exposure caused morphological changes during the formation of fungi structure (conidia and hyphae), possible resulting in ruptures of the fungal cell membrane and growth reduction (**Figure 2**).

Hyphae mortality: The O_3 gas treatment also was effective for hyphae mortality in the concentration of 60 µmol/mol treated for 120 min. The percentage of hyphae mortality after O_3 gas exposure was the highest in *F. graminearum* (97%) and *P. citrinum* (96%), followed by *F. verticillioides* (77%), *A. flavus* (51%), and *A. parasiticus* (49%). In the Control strains, the percentage of hyphae mortality were very low for *F. graminearum* (1%) and *A. parasiticus* (3%), followed by *A. flavus* (4%), *P. citrinum* (5%), and *F. verticillioides* (7%) [3] (**Figure 3**). **ROS effects:** the O_3 gas exposure showed an increase in the ROS production in the treated hyphae, this may be related to a chemical stress caused by O_3 gas. All fungi after treatment showed a strong intensity green fluorescence inside the hyphae structure due to the intracellular ROS formation [3] (**Figure 4**).

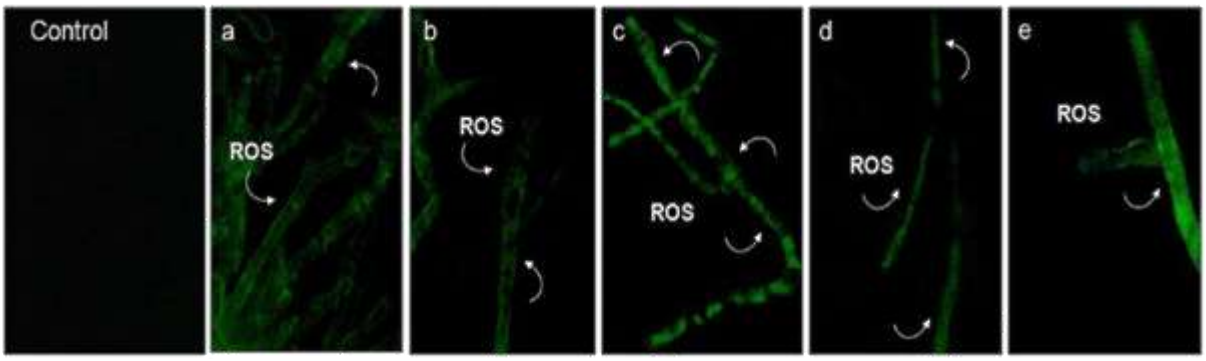

Figure 4: Reagent 2,7-dichlorohydrofluoresce in diacetate (H_2DCFDA) effect on ozone treated fungi (60 ppm, 120min) on ROS** production: (a) *F. graminearum*, (b) *F. verticillioides*, (c) *P. citrinum*, (d) *A. parasiticus* and (e) *A. flavus* [3].

MYCOTOXINS DEGRADATION

Apart from fungi, also treatments with O_3 gaseous have demonstrated efficacy on reducing mycotoxins food contamination. They were mainly reported in cereals, pulses and nuts (Table 2). Most of them presented AFLs degradation after being O_3 treated and only a few reports against other toxins producedeither by field (DON, FBs) and storage (CTR, PTL) fungi**.**

(a) Effects against toxins in food

(a.1) Aflatoxins: regarding CEREAL andO_3 treatment against AFLs, they were mainly for maize [39, 53, 55] and wheat [4, 5, 91, 92]. Maize had reduction of 66.9, 59.8, 80.8 and 23.5% of AFB_1, AFB_2, AFG_1 and AFG_2, respectively, at O_3 20 %wt and 5 min exposure [109]. Other work carried in maize had reduction of 95% of AFLs total (AFL_{total}) at 200 mg/min and 92 h exposure[55]. Prudente and King [110] applied 12 %wt of O_3 in maize during 96 h and observed a reduction of 92% of AFB_1, resulting in final contamination of less than 2 µg/kg. After 60 µmol/mol O_3treatment, Savi and others[5] showed that the AFLs levels were significantly reduced to 12.51, 41.06, 47.96 and 37.81 µg/kg after 180 min (Control group - 231.88, 265.79, 239.92 and 199.44 µg/kg), which corresponded to 94.6, 84.5, 80.0 and 81.0% of AFB_1, AFB_2, AFG_1 and AFG_2 reduction, respectively. On the other hand, after 40 µmol/mol O_3 treatment at the same exposure time, only AFB_1 and AFB_2 were significantly reduced to 43.78 and 68.79 µg/kg (88.6 and 74.8%). Additionally, it is possible to say that the $AFB_1$and AFB_2 were the mycotoxins that presented the best results regarding the two concentrations (40 and 60 µmol/mol) treatment. On the other hand, for PULSES, only studies on contaminated peanuts, O_3 AFL decontamination were carried out [83, 86, 88, 90, 96, 111]. Although soybean is the main pulse produced worldwide, no study has been carried out on O_3 to date to our knowledge against mycotoxins. However, peanuts were in deep, especially due to its mycotoxin (AFLs) most prone contamination. Dwarakanath and others [88], applied 0.025 g/min of O_3 (60 min) in contaminated peanuts (AFB^1: 82 ppb) and obtained 78% of reduction, remaining only 18 ppb of AFB^1. Application of O_3 (4.2 wt%) promoted reduction of up to 80% in artificial contaminated peanuts (AFB_1: 20 ng/g) [83]. More recently several authors reported application of different gas concentration (from 6.0 to 89 mg/g) and obtained from 25 to 94.5% reduction in the AFB_1contamination [86, 90, 96, 111]. DRY FRUITS , as they are prone to fungi infection (especially if the fresh fruits are of low quality - fungi deteriorated) toxin contamination is expected and several studies have been reported in the literature [89, 95, 101]. In a study carried out by Zorlugenç and others [95] authors observed O_3 application at rate 13.8 mg/l in dried figs during 180min and reported AFB_1 reduction of 95.2% (**Table-2**).

(a.2) Deoxynivalenol: Regarding field toxins and O_3 gas treatment, DON and FBs were studied, mainly on wheat [3, 112] in spiked DON maize [113] and in culture media too [3, 112, 114]. Li and others [112] reported that, O_3treated scabbed wheat, had 93.6% of DON degraded and the method applied was more sensitive under high moisture at concentration of 10 ml/l during 4 h. In addition to the gaseous O_3, a work carried out, utilizing aqueous O_3 though, against the trichotecenes toxins (DON, 3-acetyl DON, 15-acetyl DON, diacetoxyscirpenol, fusarenon, HT-2 toxin, 15-monoacetoxyscirpenol, neosolaniol, T-2 triol and verrucarol) reported being able to identify the degradation compounds including different intermediary products formed [115]. Savi and others [4] showed that the DON levels reduced in the wheat grains, as they were exposed to O_3 treatment. Regarding the grain pericarp (O_3 exposure at 120 min of 60 µmol/mol),the DON levels were significantly reduced. The same effect was present in the grain endosperm, although at different magnitudes (O_3 gas had a greater impact on the wheat grain external part than in the endosperm, where DON may not easily be eliminated).

(a.3) Others toxins: apart from AFLs and DON, also, FBs (FB_1, FB_2, FB_3 and hydrolyzed FB_1),CTR.

and PTL were studied to date in wheat, maize and apple juice for O_3 decontamination[5, 63,116]. In study of Savi and others[5], the CTR levels reduced after O_3 treatment at both concentrations (40 and 60 µmol/mol) after 180 min of exposure, i.e., 29.4 and 75.3% reduction. The treatment at 60 µmol/mol showed the best results, as it substantially reduced after 30 min of exposition when compared to Control Group.

(b) Mechanisms of mycotoxin degradation by O_3

The O_3 mycotoxins degradation reaction occurs due to its oxidative action on their specific toxicity site (double bounds). It includes further aromatic rings opening leading to total degradation or causing chemical modifications, thus, reducing toxicity to low levels or becoming nil [31, 90, 109, 114].

(b.1) Aflatoxins: The furan portion of AFB_1 & AFG_1 is considered to be the foundation of both toxic and carcinogenic activities, being the double bond (C_8=C_9) the toxicity determinant site. Therefore, that bond removal is the major goal of detoxification. By applying O_3, it reacts with that AFLs bond (through an electrophilic attack Criegee mechanism based) forming a vinyl ether (at the terminal furan ring) and an intermediary compound is produced (AFL ozonide) which suffers further degradation into non-toxic compounds (carboxylic acid, aldehyde, ketone and carbon dioxide) (**Figure 5**). Any O_3 in excess is fast self-decomposed and so no residue remains in food [51, 109, 117-119]. Recently, two Chinese scientist groups evaluated AFLs degradation by O_3 (utilizing a more accurate and sensitive equipment) in naturally contaminated (peanut and maize) samples with initial levels of 200 and 83 µg/kg, respectively [53, 86]. Chen and others [86] studied the AFLs detoxification mechanism in peanuts when exposed to O_3 by LC and their findings corroborate to those of McKenzie and others [109].

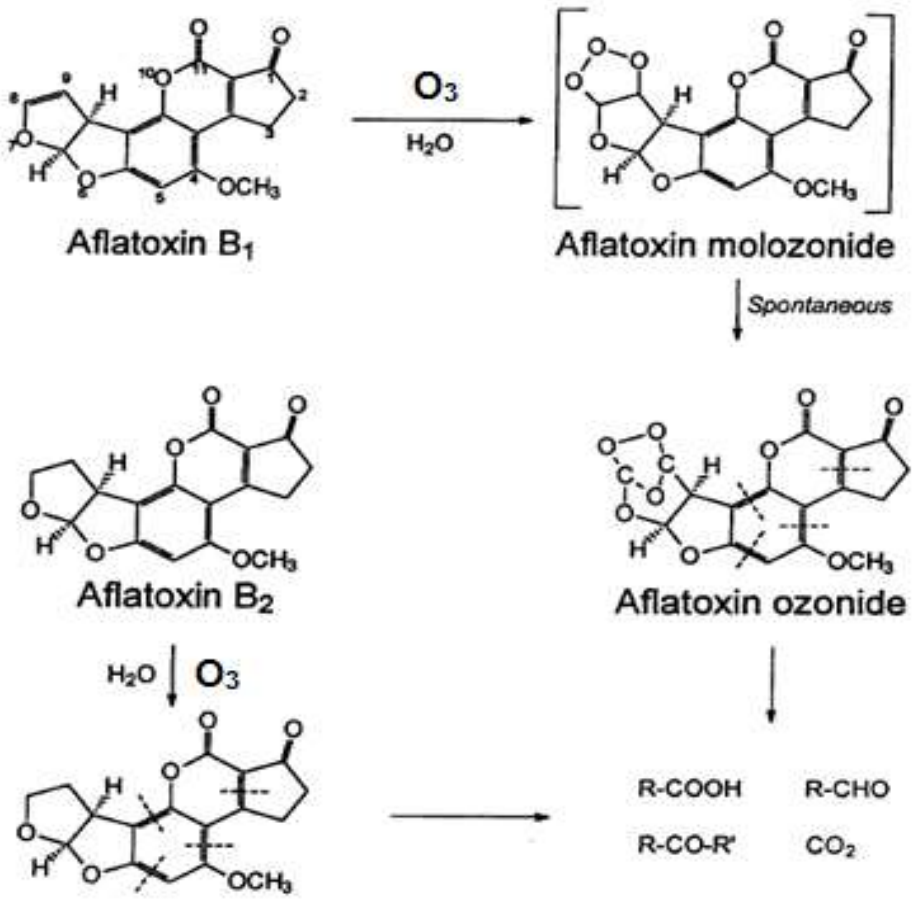

Figure 5: Degradation of aflatoxin B_1 and B_2 by ozone[109].

Indeed the dihydrofuran rings double bonds of AFB_1 and AFG_1 structures are more easily attacked by the gas which convert them into non-toxic compounds (the acids, aldehydes and ketones). Luo and others [120] as McKenzie and others [109] studied maize and confirmed that the degradation begins at the same AFB_1 double bond position with O_3 addition by LC/QTOF. The authors predicted the intermediaries (molecular formulas) formed from O_3 treated AFB_1. The toxicity of the AFB_1 degradation compounds (from naturally contaminated peanuts and maize) were tested (on turkeys, mice, and human liver cells) and proved that their toxicity reduced to nil [55, 121].

Spiked *versus* naturally AFL food contaminated: some literature reports that the efficiency of O_3 in theAFB_1 inactivation in artificially (spiked) contaminated food products are higher than those from naturally contaminated [83, 96]. They explain that data obtained in artificially contaminated food products are better because the AFLs are found only on its surface with a more uniform distribution, easier for the O_3 gas to react. While in naturally contaminated products, the AFLs may be present, apart from surface, also within the product structure (pericarp, *testae*, between cotyledons/germen) as it occurs primary with the fungi spore growth (aflatoxigenic) occurring from surface to inside the food product with subsequent AFL synthesis - leading to a heterogeneous distribution [90]. Despite that, a number of works studied with naturally contaminated food [2,85,120] reaching successful O_3 decontamination.

(b.2) Deoxynivalenol: the O_3 DON mechanism of degradation occurs as for AFLs, i.e., by attacking the double bond (C_1=C_3) leading to ozonide formation. Being an unstable molecule, its further disintegration leads to non-toxic compounds (carbonyl, carboxyl and/or ketones) [31]. Tiwari and others [114] showed the DON O_3 treated degradation and products formed (**Figure 6**).

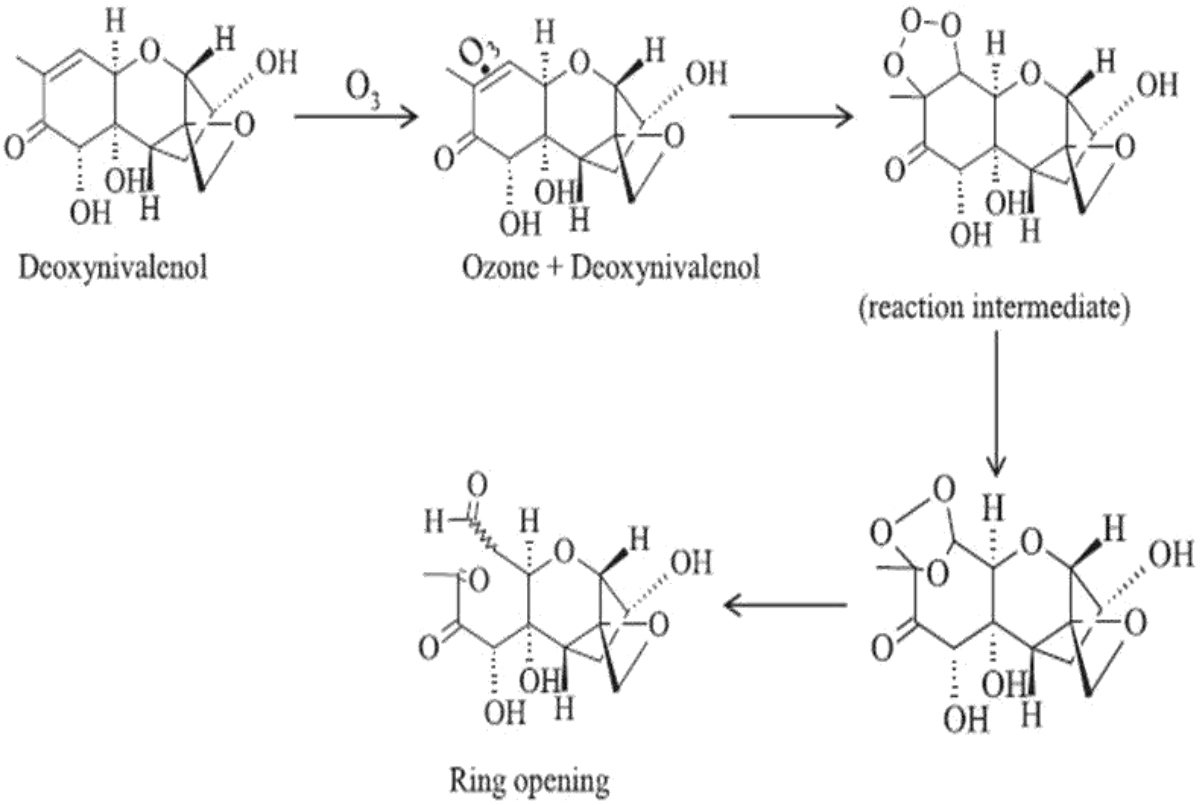

Figure 6: Degradation of deoxynivalenol by ozone[114].

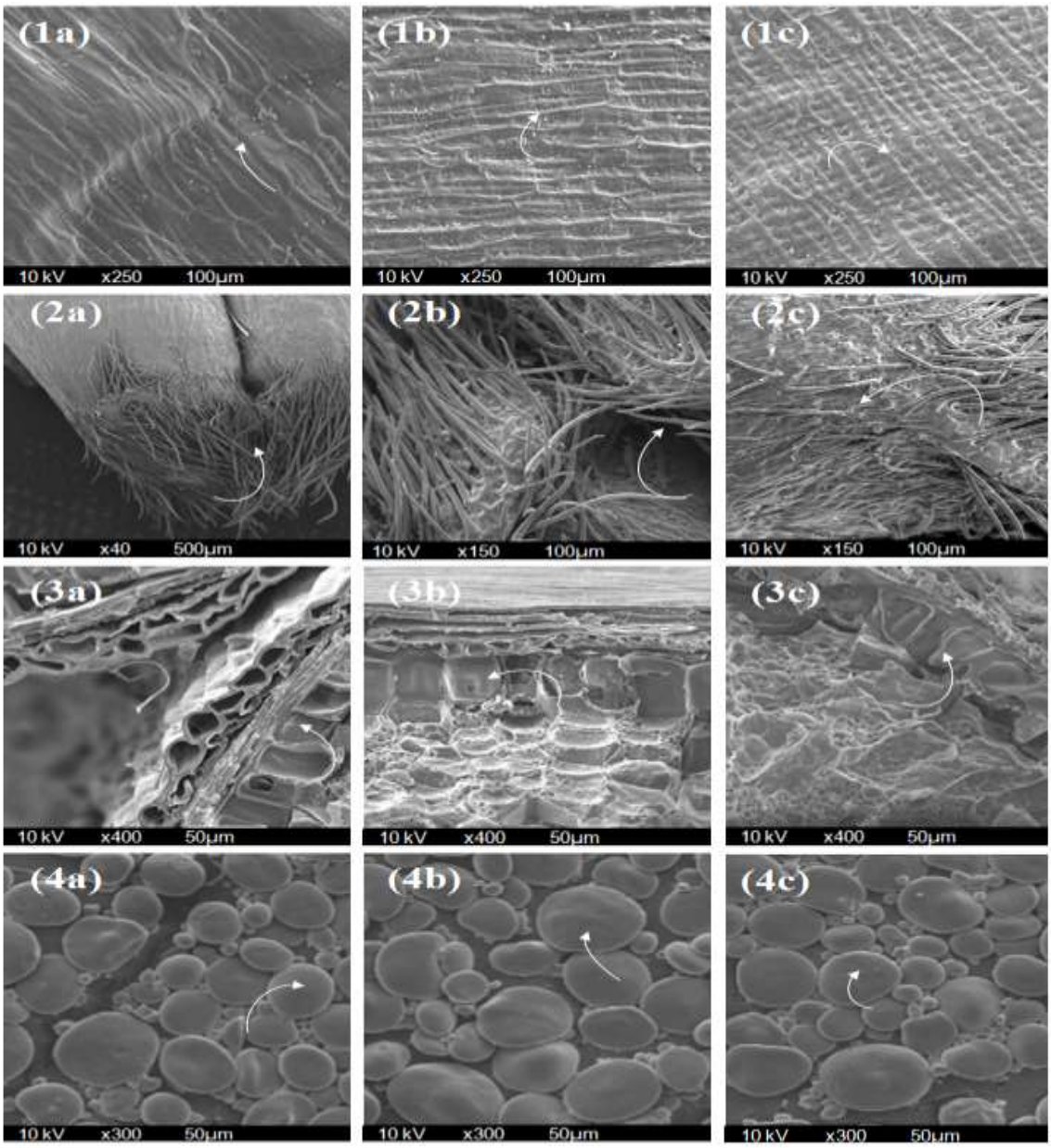

Figure 7: SEM images of microstructure characteristics of wheat grains: (A) Control and treated with 60 µmoL/moL ozone treatment - for (B) 120 min and (C) 180 min on pericarp (1) external surface (B/C); (2) brush; (3) internal surface and (4) isolated starch [4]

In addition, Young and others [115] evaluated DON O_3 degradation in aqueous solution, which the authors reported also intermediary products formed. Regarding gas and aqueous O_3 effectiveness, it is important to emphasize that gas application leads to much more contact to food components than aqueous. Moreover, it does not increase moisture (which induces microorganisms' growth and/or food degradation reactions to take place or a new step to be added - drying - during food be submitted). Inclusive gas gets deeper into food from surface, reaching hidden areas (interstice) where the fungi spore also may be found, making it easier and more efficient to be destroyed.

EFFECT OF O_3 TREATMENT ON FOOD COMPOSITION AND SEED GERMINATION

Food composition

Regarding the effect of O_3 on food composition (either raw or processed), studies have reported none or quite low O_3 interference on chemical components which is what one would need to know regarding commercial application and food quality [4, 33, 122-125].

(a) Starch - in a study of Savi and others [4], only the highest O_3 concentration (60 µmol/mol at 180 min) was able to increase the carboxyl content of treated wheat starches. Nevertheless, the O_3 treatment did not cause alterations in the starch crystallinity. The same findings were shown by Sandhu and others [124] utilizing low concentrations of O_3 (1.5 µmol/mol at 30 and 45 min), which did not produce significant difference in the starch crystallinity treated and the Control [4, 124, 125].

(b) Lipid - Savi and others [4] reported that the lipid peroxidation results showed no significant ($p>0.05$) differences between the Control and the O_3 treated wheat samples at 60 ppm for 180 min. Crowe and others [126] investigated the influence of aqueous spray treatments of 1 mg/l and 1.5 mg/l O_3 on the microbial and chemical quality indices of Atlantic salmon fillets and analysis indicate that O_3 concentration did not significantly affect the fish oil oxidation.

(c) Protein - in a study of Savi and others [4], SDS– PAGE (reduced and non-reduced) were used to analyze the changes on protein pattern in O_3 gas exposed wheat grains (60 ppm for 180 min). There was no major differences in most of the visible bands in the Black Test, Control and Treated Groups. Similar studies on proteins were also carried out by Cataldo[122, 123] and Perry and others [127].

(d) Vitamins, ferulic acid, phytic acid: Apart from carbohydrates, lipid and proteins, wheat grains O_3treated(generated *in situ*) for decontamination either for insects, fungi, bacteria, mycotoxins, pesticides, had their grain checked whether the treatment might induce alterations (on vitamins, ferulic acid and phytic acid) and no significant difference were detected between grains O_3 treated and untreated [128].

(e) Fatty acids & amino acids composition and the characteristics of wheat milling & baking: studies of the O_3 flow through a column of 3 m wheat was carried out by Mendez and others [33]. Authors reported that as the O_3 flow rate increased (from 0.02 to 0.04 m/s), a deeper gas penetration on wheat was facilitated and O_3 treatment during 30 days (50 ppm) did not produce any adverse effect on fatty acids and amino acids composition. The same was reported on the characteristics of wheat for milling and baking. It should be noted that some modifications (deformations and ruptures) may occur in the grains structure after exposure to the oxidizing agent, however, the SEM images of wheat grains microstructure characteristics did not demonstrate apparent damage caused after O_3 treatment at 60 ppm for 120 and 180 min [4]. The external and internal surface of pericarp grain, brush pericarp and isolated starch were intact when compared to Control Group (**Figure 7**).

Seed germination

Regarding the O_3 effect on wheat grain germination behavior, it was observed slight germination capacity reduction (12.5%), no modifications on seeds coleoptile length and on the seminal root (up to 180 min of gas exposure at 60 µmol/mol concentration). In addition, even after O_3 gas treatment (60 µmol/mol) at a shorter time of exposure (120 min), no effect on germination was observed [4] (**Figure 8**). According to Wu and others [57], by applying different O_3 doses (0.016, 0.065, 0.16 and 0.33 mg/g wheat/min), no effect on wheat germination was observed even after 60 min of exposure to that gas. However, at the concentration of 0.98 mg/g wheat/min, the germination rate

reduced to 61.3% at 45 min of exposure to O_3. It is important to emphasize that effective inactivation

of fungi and mycotoxins had already been achieved far below the thresholds for germination reduction, and therefore showed to be an effective method for stored wheat grains protection.

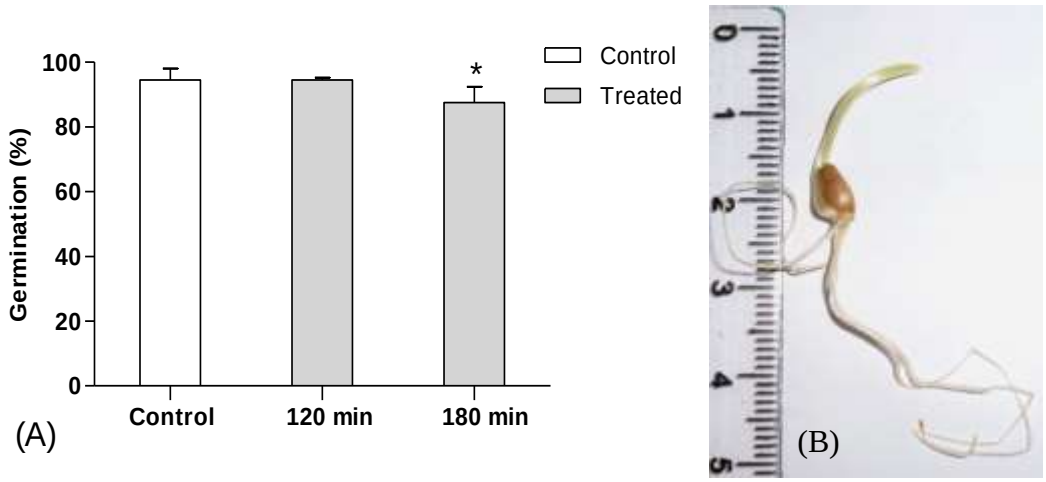

Figure 8: Wheat germination after ozone (O_3) gas treatment (A) percentage at 60 µmol/mol for 120 and 180 min [symbol indicate statistically significant when compared to Control *p<0.05 by Tukey test]; (B) germinated seed [60 µmol/mol for 180 min] [4].

CONCLUSIONS

O_3 is a strong oxidant and an effective alternative to traditional processes against a variety of fungi genera and their toxins apart from pesticide residues and other contaminants. Total fungi load, as high as 10^6 CFU/g, can be efficiently destroyed by O_3 gas (at 1.0 ppm), and that reduces the problem in a broad range of raw and processed food. In addition, toxigenic fungi have been demonstrated being destroyed in a variety of foods (barley, maize, wheat, rice, dates, figs, Brazil nuts, peas, peanuts, dry fruits) by O_3 gas, with concentrations ranging from 0.3 to 5,000 ppm (mean 40 to 100 ppm) and exposure times from 5 min to continuous (mean 40 to 60 min). Mycotoxins (AFLs, FBs, DON, PTL and CTR) are destroyed by that gas at adequate conditions (concentration and time of exposure) as long as they are adjusted to the characteristics of food to be decontaminated and the toxin level. Regarding fungi genera and species, *Fusarium* followed by *Penicillium* and *Aspergillus* are the most efficiently O_3 destroyed and studied to date. Despite of data reported on that gas efficiency in different contaminants (insect, fungi, yeast, toxins and pesticides) in food, there is a need of studies on its application in larger and adequate installations for effective O_3 application. Important factors to be considered are the materials properties to be treated (surface roughness and imperfections, presence of mechanical damage, material permeability, porosity) and also the environmental conditions (relative humidity and temperature).

COMPETING INTERESTS

Authors declare no conflict of interest.

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***Corresponding author: Vildes M. Scussel:** Laboratory of Mycotoxicology and Food Contaminants, Food Science and Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, Rod. Admar Gonzaga, Itacorubi, 1346, Florianopolis, SC, Brazil. www.labmico.ufsc.br.