The Journal of the Science of Food and Agriculture, 2018,2(2), 35-39
http://www.hillpublisher.com/journals/jsfa

Ozone Effect to Control Lemon Postharvest Diseases in Storage Chamber

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How to cite this paper: Sepúlveda, T.M., et al. (2018) Ozone Effect to Control Lemon Postharvest Diseases in Storage Chamber. *The Journal of the Science of Food and Agriculture*, *2(2)*, 35-39.
http://dx.doi.org/10.26855/jsfa.2018.02.002

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Abstract

Ozone was extensively evaluated to control postharvest pathogens and its use presents commercial interest since it is considered an innocuous substance free of residues. In Tucuman (Argentina), research carried out on this technology in citrus is scarce. In this paper the ozone effectiveness to control citrus green mold (*Penicillium digitatum*) (*Pd*) was evaluated. *In vitro* and *in vivo* tests were conducted in storage room. Petri dishes with potato dextrose agar (PDA) with and without Pd disks, were exposed to ozone (0.05 ppm) at 5 and 12 ℃, at two different height of 0.10 and 1.5 m, for 8, 24 and 48 hs. Wounded and artificially inoculated lemons were kept in the same storage room. In other tests, artificially inoculated lemons were stored in three open boxes and six export boxes, separately. Fruit were evaluated on the 7th, 14th, and 21st days after treatments. These same treatments were conducted in another room without ozone as control. The average diameter and the number of *Penicillium digitatum* colonies grown *in vitro* were inversely proportional to the ozone exposure time, showing that this gas reduced the environmental contamination after 24 hs of exposure. The incidence of the disease in inoculated lemons was similar in both storage rooms, whereas in wounded fruit the infection was inversely proportional to the exposure time to ozone. Results showed that disease severity was low when ozone was used, even though the incidence was high. The ozone static effect was higher when fruit was in direct contact with the gas.

Keywords

Lemon, ozone, Green mold.

1. Introduction

The Ozone, an oxygen triatomic form, was recognized as innocuous substance (GRASS) to apply in foods in USA, in 1997 (Graham et, al 1997 and US –FDA, 1997)
Since the interest to use this gas in food industry was increased due to the fact that itis a substance present in atmosphere, highly oxidant with a strong sanitation effect over a wide range of microorganism (Kadre et al, 2001). Other advantage of Ozone employed is that it does not generate chemical residues, therefore it is well accepted by the majority of the organic consumer organizations.
In order to treat postharvest fresh fruits and vegetables, Ozone can be applied in continue or intermittent atmosphere of storage chambers or in water as a sanitizer of surfaces. (Palou et al., 2003).
There are numerous reports about the use of this gas (Klotz, 1936; Hopkins et al., 1949; Harding, 1968; Palou et al., 2001, 2002) to control Green mold. Palou et al reported in 2001 that ozone continuous application at low doses (0.3 or

1.0 ppm) in cool storage chambers did not reduce the ultimate incidence of this pathogen. However, on wounded and artificially inoculated fruits stored in ozonized cool chamber *P. digitatum* grew more slowly than fruits stored in cool chamber without the gas. Furthermore, in 2003, Palou et al. reported that the use of ozone during storage to treat fresh fruit diseases is limited by the type of ventilation used in packing house and container.
The objective of this paper was to evaluate the effect of Ozone (0.05 ppm) on *environmental microorganism*, and on wounded lemons artificially inoculated with *Penicillium digitatum*, and placed into different boxes stored at 5 ℃.

2. Materials and methods

2.1Ozone application system

Tests were carried out at the Experimental Station in INTA-Famailla. An ozone generator provided by ECOTRES TECNOLOGIA AMBIENTAL COMPANY, was installed in a 62.5 m^3chamber at 5 ℃ constant temperature. The chamber is equipped with a 40 m^3/h turbine capacity Ozone concentration used was 0.05 ppm (vol/vol) relative humidity 90% (Palou, et al., 2002). Control treatments were carried out in other storage chamber set at the same conditions except ozone.

2.2 Ozone effects on the growth of environment microorganism

Ambient microorganisms grown on PGA (potato glucose agar) medium exposed for 8, 24 and 48 hours in ozone chamber were evaluated. After that, exposed plates were incubated at 24 ℃ during 4 days, and visually evaluated by counting colonies. Values correspond to an average of quadruplicates.

2.3 Ozone effect on P. digitatum growth in vitro

P. digitatum mycelium disks (0.5 cm diameter) of 7 to 14 days, were placed individually in the middle of a Petri dish with PGA medium, and incubated for 18 hours at 24 ℃. Then the plates were exposed to ozone in the chamber during 8, 24 and 48 hours. Then, they were removed from the chamber and incubated at 24 ℃. Colony radius was determined after 4 days and values correspond to an average of quadruplicates.

2.4 Conidial suspension, fruit samples and fruit inoculation.

Conidial suspension of 1×10^6 conidia ml^{-1} was prepared using as described by Cerioni et al. (2012). *P. digitatum* isolates were cultured for 7–14 days on potato dextrose agar (PDA, Difco Laboratories, Detroit) at 25◦C. For fruit inoculation, 'Eureka' lemons (Citrus limon (L.) Burm) collected from commercial orchards were employed in lab, and used within the 24 h after harvest. Fruit were inoculated by wounding once with a stainless steel rod (1 mm wide and 2 mm in length) immersed in the conidial suspension.

2.5 Ozone effect on P. digitatum growth in vivo

In the first trial, 25 artificially inoculated lemons were placed in open boxes and exposure to ozone. The fruits were removed from chamber with ozone after 8, 24 and 48 hours. Then, these fruits were incubated at 24 ℃ and 90 % RH, and visually evaluated after 4 days. In the second trial, 25 artificially inoculated fruits were placed in 3 open boxes, after 18 hs of incubation. For the experiments three export boxes (standard corrugated fiberboard citrus cartons) were used; in each box 6 artificially inoculated fruits were intercalated with health fruits. Open and export boxes were exposure in ozone chamber for 7, 14 and 21 days. Disease incidence was evaluated visually.

2.6 Statistical analysis

An analysis of variance (SAS Institute Inc., Cary, NC, USA) was applied to the transformed data and means were separated by Fisher's Protected Least Significant Difference test (LSD, $P = 0.05$).

3. Results and discussion

3.1Ozone effect on ambient microorganism growth
A continuous exposure to 0.05 ppm of ozone during 8 hours had an adverse effect on the growth of the three types of microorganisms evaluated (Table 1)

The increased of the number of colonies of bacteria at low temperatures in chambers without Ozone, indicates that low temperatures do not affect their growth.

Table 1. Influence of continuous exposure to 0.05 ppm ozone at 5 ℃ on growth environmental microorganism. Average number of *Penicillium spp*., other fungus and bacteria colonies counted on plates after different exposure time.

	Chamber 5 ℃			Chamber 5 ℃ + OZONE		
	8 hs	24 hs	48 hs	8 hs	24 hs	48 hs
Penicillium spp	29.2 A	24.8 A	9.8 B	3.2 BC	0 C	0 C
Other Fungus	41 B	90 A	55.8 B	1.8 C	0.2 C	0 C
Bacteria	6.8 C	21.4 B	36.8 A	0 C	0 C	0 C

Numbers correspond to average of 4 replicates. Different letter means highly significant difference according test LSD Fisher α= 0.05.

Hibben and Stotzky (1969) reported that fungi spore sensibility to oxidant ozone action is influenced by the fungus species, morphology, substrate, doses and type of contact with the gas. Our results showed that all the fungi species present in the chamber environment were effectively controlled by ozone exposure; being those of the *Penicillium* genus slightly less controlled at 8 hours. These results indicate that Ozone is an excellent alternative to apply in fruit and vegetables storage chamber as a sanitizing agent.

3.2 Ozone effect on P. digitatum growth in vitro

The continue exposition to ozone (0.05 ppm) had negative effect on the growth of *P. digitatum* mycelium discs. However a decrease of the radial growth of mycelium was also detected in the room without Ozone, as consequence of the low temperature (5 ℃) (see Table 2). When these plates were removed from the ozone chamber to incubation condition (24 ℃), the mycelium was observed after 48 hours. Similar results were reported by Klotz (1936), who reported that ozone partially inhibited *P. digitatum* germination and growth but the culture recovered when removed from ozone exposure, even after three weeks of continuous exposition. In this study we did not evaluate that long exposure time due to dehydration of culture medium after 48 hours. Our results agree with those previously reported by Palou et al. (2001).

Table 2. Ozone effect on the colony growth of *Penicillim digitatum*. Colony diameter (cm) after 8, 24, 48 hs continuous exposure to ozone (0.05 ppm and 5 ℃).

Penicillium digitatum	Chamber 5 ℃			Chamber 5 ℃ + OZONE		
	8 hs	24 hs	48 hs	8 hs	24 hs	48 hs
	1.66 A	1.32 AB	1.06 B	1.46 AB	1.18 B	0.42 C

Diameter average were obtained from 4 replicates. Different letter means highly significant difference according to test LSD Fisher α= 0.05**.**

3.3 Ozone effect on P. digitatum growth in vivo

There were no infections or pathogen development associated with fresh citric fruit on wounded fruits, when they were exposed at low temperature plus ozone. However, lemons exposed to ozone for 24 and 48 hs were not infected by *P. digitatum* after incubating at 24 ℃ for 4 days (Table 3). This result could be due to the reduction of *Penicillium* spp colonies number reported in Table 1. Results suggest that Ozone has a fungistatic effect over artificially inoculated fruit after 48 exposure, because when fruits were removed from the exposition to the gas and were incubated at 24 ℃ during 4 days, 100 % of lemon infection were observed. Similar results were reported by Palou et al (2001), who observed that when fruits were continuously exposed to ozone for a week the green mold infection was slow but at the end of this storage period the disease incidence did not reduce. Due to restricted ozone penetration power probably spore and subsequent fungi mycelium were protected from gas oxidation action.

Table 3. Influence of continuous exposure to 0.05 ppm ozone on incidence of artificially wounded and inoculated Eureka lemons. *P. digitatum* infection percentage at three times exposure and not exposed to ozone, before and after incubated at 24 ℃.

	Before storage at 23 ℃/ 4 days				After storage at 23 ℃/ 4 days			
% *Pd*	Chamber 5 ℃		Chamber 5 ℃ + OZONE		Chamber 5 ℃		Chamber 5 ℃ + OZONE	
infection	Inoculated	Wounded	Inoculated	Wounded	Inoculated	Wounded	Inoculated	Wounded
8 hs	0	0	0	0	96	40	100	64
24 hs	0	0	0	0	100	12	100	0
48 hs	65	0	10	0	100	0	100	0

Inoculated fruit placed in open and export boxes had similar behavior and significant difference in delaying infection growth until 14 days in ozone chamber. While in the chamber without ozone both boxes achieved 100 % disease incidence after 21 days, in the chamber with ozone the Pd incidence was 92 % in open boxes and 80 % in export boxes (Table 4). Nevertheless the disease severity was different in each type of box: whereas the infected fruit without ozone were completely sporulated ,in infected fruit exposed continuously to the gas, only aerial mycelium was observed and in some cases incipient infection (data not showed). Same results were reported by Palou et al. (2001) and Harding (1968) who observed that in infected fruit the sporulation could be controlled meanwhile the ozone was present, but it reappeared again when fruits were removed from gas. However, our results disagree with the results reported by Palou et al. (2001) and Harding (1968) because after 21 days in ozone chamber we observed a decrease of the disease incidence in export boxes as compared to open boxes.

Table 4. Influence of continuous exposure to 0.05 ppm ozone on incidence of artificially inoculated Eureka lemons in open or export boxes. *P. digitatum* infection percentage at three times exposure and not exposed to ozone.

% *Pd*	Chamber 5 ℃		Chamber 5 ℃ + OZONE	
infection	Open Boxes	Export Boxes	Open Boxes	Export Boxes
7 d	88 A	90 A	8 C	20 C
14 d	100 A	100 A	72 B	70 B
21 d	100 A	100 A	92 A	80 B

Numbers correspond to average of 3 replicates. Different letter means highly significant difference according test LSD Fisher α= 0.05.

In conclusion, our results show that a continuous application of 0.05 ppm as gaseous ozone in storage chamber delays the growth, and the disease severity caused by *P. digitatum*, but does not reduce its incidence. These results suggest that this technology cannot be considered as a substitute of synthetic fungicide applied at packing fruit. The continuous ozonization in chamber reduced inoculum load of present ambient microorganism, generating an interest of usage in the storage of numerous fruit and vegetables. In citrus, ozone inhibited the growth of aerial mycelium and sporulation of *P. digitatum* avoiding that infected fruits may become a source of inoculum and contamination of other fruits placed in the same boxes or bins destined to repack. Nevertheless should be kept in mind that ozone effect is only transitory and pathogens grow as soon as the gas is eliminated. Moreover, since the gas cannot penetrate through neither plastic nor cardboard its effect is reached when fruits are stored in packing open surfaces. Furthermore, due to its high oxidant power, ozone can be harmful for human, phytotoxic to fruit and corrosive to many materials, therefore its concentration must be automatic strictly controlled to guarantee safe conditions to operators.

Acknowledgments

We thank to San Miguel SA, INTA and ECOTRES Tecnología Ambiental.

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