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Effect of ozone treatment on postharvest disease and quality of different citrus varieties at laboratory and at industrial facility

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Abstract

The effects of continuous and intermittent (simulating a day-night cycle) exposure to ozone enriched atmosphere (from 1.6 to 60 mg kg^{-1}) at 5 ºC for 28 d and subsequent shelf life at 20 ºC for 15 d on six citrus varieties (two mandarins: Fortune and Ortanique; and four oranges: Navelate, Lanelate, Salustiana and Valencia) were investigated. *In vitro* and *in vivo* growth of *Penicillium digitatum* and *Penicillium italicum* was first assessed. Based on the results obtained, continuous 60 mg kg^{-1} ozone and intermittent 1.6 mg kg^{-1} ozone were selected for industrial trials, and decay and

oleocellosis incidence, colour, firmness, weight losses and juice (content, soluble solids, pH, titratable acidity and vitamin C) were analysed. Results showed that the application of ozone was not detrimental to fruit quality. Furthermore, the application of both continuous and intermittent ozone delayed decay and oleocellosis incidence and slowed down the development of the colouring process, while reducing firmness and weight losses. For industrial applications, the advantage of using ozone 12 h d^{-1}, simulating a day-night cycle, is that workers would not be exposed to ozone inside the cold storage room during the day shift.

Keywords: Mandarins; Oranges; *Penicillium digitatum*; *Penicillium italicum;* Shelf life

1. Introduction

Citrus industry, including harvesting, handling, transport, marketing and delivery, provides nowadays millions of jobs in more than 137 countries around the world. Citrus ranks first among fruits in the world, due to their huge annual production (90–110 million tons) (Karaca, 2010).The importance of citrus arises from their nutritional and antioxidant properties. However, this nutrient composition along with their higher water content makes them susceptible to infection by microbial pathogens, mainly fungi because citrus fruits are quite acidic, from harvesting to consumption (Talibi et al., 2014). Economic losses caused by fungi are especially damaging for citrus fruit and may even reach 5 to 10% of the total production, thus limiting the overall profitability of the citrus industry. A number of postharvest diseases are responsible for citrus losses during storage and shelf life. Some of them are the result of preharvest infections while others are produced during postharvest, mainly due to injuries. *Penicillium digitatum* (green mold) and *P. italicum* (blue mold) are the major pathogens of citrus fruits. The source of both fungal infections is practically continuous during the season, and can

infect citrus in the grove, the packinghouse and during distribution and marketing (Palou et al., 2008). Traditionally, these losses have been efficiently controlled by the application of fungicides, including benzimidazole (thiabendazole, benomyl and carbendazim) and sterol inhibitors (imazalil, prochloraz and propiconazole) sodium orthophenyl phenate and different mixtures of these compounds (Eckert and Brown, 1986; Eckert, 1990; Palou et al., 2008; Talibi et al., 2014). The postharvest use of these fungicides is subject to registration and permission in various countries (Talibi et al., 2014). However, consumers are becoming increasingly aware of the fact that many of these chemical treatments represent a potential risk for human health and environment. The application of these substances during the postharvest manipulation of the fruit can lead to the presence of certain residual quantities of these chemical products or their metabolites, potentially harmful for human health, in the treated fruits. The residuals of the fungicides used before or after harvest may also contaminate the environment. Furthermore, the chemicals used become inefficient, due to the selection of resistant pathogenic stocks. A vicious circle begins: Once resistance has been developed in the pathogens, new, more effective chemicals have to be produced to maintain the same level on the control of produce decay, which leads to the increase of the risk of possible toxicity for environment and human health, until once again resistant pathogens occur, and so on. For these reasons there has been an increasing interest in the development of effective, non-harmful physical procedures for decay control of horticultural products, including physical treatments (curing, hot water and irradiation treatments), chemical treatments (sodium bicarbonate, calcium polysulfide and ammonium molybdate treatments, borax baths, and addition of natural compounds such as volatiles and essential oils, plant extracts, peptides, proteins, chitosan and chitosan derivatives), biological treatments (utilisation of microbial antagonists, application of naturally

derived bioactive compounds, and induction of natural resistance) and combinations of the above-mentioned treatments for integrated disease management (García et al., 2016).

Ozone, the three atomic form of oxygen, is a gas with a strong oxidant capacity that was granted GRAS (Generally Recognized As Safe) status in 1997 by the U.S. Food and Drug Administration and approved for use as disinfectant or sanitizer in food processing (Karaca, 2010). Its main advantage is that ozone does not present safety concerns about consumption of chemical residues in the treated produce so it is accepted by many organic grower organizations (Horvitz and Cantalejo, 2014). Ozone may therefore constitute a non-contaminant alternative for reducing the use of fungicides during fruit storage. Although high concentrations show toxic effects and predispose the vegetables to *Botrytis* infection (Wukasch and Hofstra, 1977), ozone used at a suitable level in the storage atmosphere may provide disease protection with a minimum of physiological damage (Liew and Prange, 1994). Such atmospheres delay mycelial growths of *Sclerotinia sclerotiorum*, *Botrytis cinerea* or *Rhizopus stolonifer* (Liew and Prange, 1994; Sarig et al., 1996, Tzortzakis et al., 2007). Furthermore, ozone may induce resistance of plants to pathogens (Kangasjärvi et al., 1994). With the aim of reducing decay incidence in citrus and other fruits, ozone in aqueous and gaseous environments has been assayed. For the former, the low solubility of ozone in water may be a hindrance for aqueous ozone applications. Thus, Smilanick et al. (2002) reported that green mold and sour rot on citrus fruit, caused by *P. digitatum* and *Geotrichum citri-aurantii*, respectively, were not reduced by 20 min immersion in 10 mg kg^{-1} ozone. On the contrary, gaseous ozone used at proper concentrations in the storage atmosphere can protect several fruits and vegetables against diseases with minimum physiological damage (Nadas et al., 2003). From the information available in literature, it can be

concluded that citrus exposure to gaseous ozone provides better results, as it will be discussed later, and thereby the use of an ozone-enriched atmosphere was selected for this research.

The objective of this work was to evaluate first the effect of continuous exposure to 0.6, 1.6 and 60 mg kg^{-1}gaseous ozone and intermittent exposure to 1.6 mg kg^{-1}gaseous ozone (day-night cycle) on the *in vitro* microbial growth of *P. digitatum* and *P. italicum* on potato dextrose agar Petri dishes and on the *in vivo* microbial growth of *P. italicum* on artificially inoculated citrus stored at low temperature. A 10^6 conidia mL^{-1} suspension was used for both *in vitro* and *in vitro* experiments. This concentration of pathogen was chosen because it has been verified that this amount is enough to provoke complete decay of citrus fruit (Eckert and Brown, 1986) and it has been already successfully tested in previous works (Nunes et al. 2007; García et al., 2016). Once the most suitable conditions for ozone treatment are found, the next step is to assess the effects of exposing citrus fruits to ozone during storage at 5 °C (up to 28 d) and subsequent shelf life at 20 ºC (up to 15 d) at industrial scale on different fruit quality parameters. The main target of this research is to check the feasibility of the application of ozone at industrial facilities, searching thereby the conditions in which decay incidence is reduced without affecting fruit quality during both cold storage and shelf life of citrus fruit.

2. Materials and methods

2.1. Citrus fruit

Six citrus varieties were used, two mandarins (*Citrus reticulata* cvs, Fortune and Ortanique) and four oranges (*Citrus sinensis* cvs, Navelate, Lanelate, Salustiana and

Valencia), which were grown in the commercial orchard "El Zumajo" located in Río Tinto (Huelva, Spain) by the company Río Tinto Fruit S.A. Each variety was harvested in the moment in that the fruit showed ≥ 90 % of skin surface degreening and the Total Soluble Solids:Titratable Acidity ratio in the juice was ≥ 10:1.

2.2. Pathogen microorganisms

The fungi *Penicillium digitatum* and *Penicillium italicum* were obtained from the Spanish Type Culture Collection and maintained on potato dextrose agar plates. Conidia of a 7–12 d culture grown at 25 °C were suspended in 100 mL sterile distilled water with two drops of Tween 80. The suspension was adjusted to 10^6 conidia mL^{-1}, using haemocytometer for each fungus. For *in vitro* experiments, PDA Petri dishes were inoculated in its geometrical centre using a 1.4 mm diameter steel rod, previously immersed in each conidia suspension. Similarly, for *in vivo* assays, fruits were wounded and inoculated on their flavedo using the same system and conidia concentration, but only with *P. italicum*.

2.3. Ozone treatments at laboratory scale

Ozone was produced by an OMD 100 ozone generator (Ozodiex S.A., Barcelona, Spain) in all the trials.

2.3.1. *In vitro* assays

In vitro inoculation at laboratory scale was performed to assess the direct effect of ozone treatment on pathogens and so determine the most suitable conditions for *in vivo* inoculation. For this purpose, 4 replicates of 10 PDA Petri dishes each one were carried out for each pathogen and treatment. After inoculating, Petri dishes were let stand for 2

h at room temperature to allow the development of the infection and immediately afterward were exposed to ozone (except for control sample, which was exposed to air) at 5 ºC for 28 d. Four ozone atmospheres were assayed: continuous 0.6, 1.6 and 60 mg kg^{-1} ozone-enriched atmospheres, and intermittent exposure (12 h on, 12 h off) to 1.6 mg kg^{-1} ozone-enriched atmosphere, simulating a nocturnal ozone application. In order to monitor the fungal growth, the diameter of the resulting fungal colony placed in the centre of each plate was measured and the results presented as the percentage of the plate covered by the pathogen at each sampling date.

2.3.2. *In vivo* assays

After harvesting, Valencia oranges were let stand for one night at room conditions (20 ± 2 ºC and 80 % relative humidity). Afterwards, 4 groups of 20 oranges each (i.e. 4 replicates) were inoculated with *P. italicum*. Two hours after the inoculation citrus were intermittently exposed to 1.6 mg kg^{-1} ozone atmosphere (12 h on, 12 h off) at 5 ºC for 28 d. Meanwhile, another 4 groups with the same number of inoculated fruits were stored at 5 ºC for 28 d in air without ozone (control samples).

2.4. Ozone treatments at industrial scale

Industrial-scale ozone treatments were carried out using citrus not previously treated with fungicides and without pathogen inoculation. Immediately after harvesting fruit of each variety were distributed in 20 perforated plastic boxes able for 20 kg of fruit and simultaneously treated in the ozone chamber. Control samples of each variety were subjected to the same procedures but using air instead of ozone atmosphere. Three ozone treatments were performed. In the first trial, Salustiana, Lanelate and Navel oranges and Fortune and Ortanique mandarins were exposed to continuous 60 mg kg^{-1} ozone-enriched atmosphere at 5 ºC for 28 d. After this period, both ozone-treated and

control fruit were located at 20 °C under air, simulating shelf life during 14 d. In the second ozone trial, other two groups of 20 perforated boxes of Lanelate and Valencia oranges were exposed to continuous 60 mg kg^{-1} ozone-enriched atmosphere or under air for up to 28 d at 5 ºC. Four boxes of each variety were randomly taken out from the ozone chamber (or air chamber for control fruit) every 7 d to assess their shelf life at 20 ºC for another 15 d. Losses due to decay and qualitative parameters of the fruit and their juices were measured at 0, 5, 10 and 15 d of shelf life. Finally, the last trial was performed with Valencia oranges only, using air or intermittently 1.6 mg kg^{-1} ozone-enriched atmosphere simulating a day-night cycle (12 h on, 12 h off) during the cold storage at 5 ºC. Similarly to the second trial, four boxes of Valencia oranges were randomly taken out from the 20 boxes originally placed in the chambers at 5 ºC (ozone or air) after 7, 14, 21 and 28 d, respectively, to assess their shelf life at 20 ºC for another 10 d. Decay incidence and qualitative parameters of oranges and orange juices were determined at 0, 4 and 10 d of shelf life. The conditions of the three experiments are summarized in Table 1.

2.5. Quality parameters

During ozone treatment and shelf life at industrial facility, both decay incidence (physiological damage) and quality parameters of the fruit were monitored. Decay and oleocellosis incidences were evaluated visually examining all fruit and calculating the percentages in relation to the totality of fruit of each one of four boxes randomly selected in each sampling date, obtaining 4 replicates each time.

Colour index (CI), strongly related to degreening and ripening stage of citrus, was calculated from the CIEL*a*b* parameters obtained by using a Minolta CR-200 handheld chromameter (Konica Minolta Inc.), with a measuring area of 8 mm in diameter, diffuse illumination and a viewing angle of 0°, as follows:

$$CI = 1000a^* L^{*-1} b^{*-1}$$

Measurements were performed on the equatorial zone of 20 citrus fruit, randomly taken from each treatment (5 fruit from each one of the four boxes).

The firmness of the same fruit used for colour determination was measured with a Zwick 3300 densimeter (Zwick GMBH & Company, Ulm, Germany) and expressed in N.

Ten citrus fruit were randomly selected from each treatment and individually weighed at each sampling date to monitor the weight losses during the whole postharvest period (cold storage and shelf life), referred as percent of the original weight.

The fruit used for colour and firmness determinations were then cut in half and squeezed, using a Sammic squeezer (Sammic SA, Azpeitia, Spain) and their juice volumes were determined independently with a 500 mL measuring cylinder. The extracted juice was expressed as mL juice per kg fresh weight. Total soluble solids (AOAC, 2005), pH, and titratable acidity were measured in the same juices obtained for the determination of juice content. The results were expressed in % total soluble solids, pH values, and % citric acid, respectively.

Ascorbic acid content (vitamin C) was estimated first mixing 10 mL juice with 90 mL of 4% oxalic acid and then titrating a 5 mL aliquot of this mixture with 2,6-dichlorophenolindophenol by measuring its reduction with ascorbic acid. Titration volumes were compared with 1 g L^{-1} ascorbic acid (Sigma-Aldrich, St. Louis, MO), and the results were expressed as g of ascorbic acid per L of juice (Hiromi et al., 1980).

2.6. Sensory analysis of the citrus juice

Sensory analysis was solely performed in the assays at industrial scale by 10 panellists. The test consisted in ordering from best to worst quality two juice samples, the ozone-

treated one and the control. The sample of best quality was scored as "1" and the worst as "2". If a panellist did not find any difference in quality between samples, then it was given a score of "1.5". The scores by the panellists for each sample were summed to obtain a value representative of the quality thereof. Panellists were asked to pay particular attention to the possible occurrence of off-flavours. Each analysis was performed in triplicate.

2.7. Statistical analysis

All the studied variables were analysed by ANOVA. For each given time, the effect due to the different treatments (air or ozone) was evaluated for each variable and considered significant with a 5% level. ANOVA was calculated by using Costat 2.10 software (Cohort Software, Berkeley, USA). Sensory analyses were analysed by Friedman's test to evaluate if the ranking obtained had statistical significance (Land and Shepherd, 1988). The effectiveness of the treatment applied on an industrial scale on decay incidence was performed using contingency tables and χ^2 test with a 5 % of significance level.

3. Results

3.1. Experiments at laboratory

3.1.1. Effects of ozone concentration on *in vitro* fungal growth

The use of different ozone concentrations during cold storage exerted a similar effect on the mycelial growth of both microorganisms (Fig. 1 and 2). Mycelial growth of microorganisms stored at 5 ºC under air or under 0.6 mg kg^{-1} ozone atmosphere started from the beginning of incubation, while that of microorganisms stored under higher ozone concentration was delayed for several days. The area covered by fungi linearly increased in all treatments; the higher the ozone concentration, the lower the slope.

Furthermore, the growth of secondary colonies was observed in PDA incubated under air (data not shown). This did not occur under ozone atmosphere. Therefore, ozone not only slowed down fungal growth but also impeded other microorganism infection. Interestingly, *P. digitatum* and *P. italicum* growths were completely inhibited when using intermittently 1.6 mg kg^{-1} ozone (12 h d^{-1}, day-night cycle) (Fig. 1 and 2).

3.1.2. Effects of ozone concentration on *in vivo P. italicum* growth

Storage under ozone atmosphere (1.6 mg kg^{-1} 12 h d^{-1}) markedly increased the lag phase of the fungus but without visible growing on the fruit surface (Fig. 3). The lag phase under ozone atmosphere was 3 times higher (15 d) than that of oranges stored solely under air (5 d). The subsequent stage of decay incidence increasing (related to the exponential growth stage of the microorganism) was also quicker in fruit stored under air atmosphere. On the other hand, the characteristic *Penicillum* sporulation was not observed once the fungus grew on the oranges when applying ozone, while the fungus took the typical green-blue tint of its spores in oranges stored under air atmosphere.

3.2. Decay incidence in ozone trials at industrial facility

Interestingly, decay losses were not detected in the first trial in any of the treatment tested, so no conclusions could be extracted from this experience (data not shown). By contrast, in the second trial in which continuous 60 mg kg^{-1} ozone was also assayed, the ozone-enriched atmosphere clearly reduced the natural decay incidence in oranges during storage at 5 °C in both Valencia and Lanelate varieties (Fig. 4). This decay incidence reduction enhanced the shelf life of the oranges at 20 ºC (Fig. 5), regardless the previous period of ozone exposure (7, 14, 21 or 28 d). Lanelate oranges showed higher initial decay incidence than Valencia oranges. As a result, their decay incidence

was higher during cold storage under both ozone and air. Finally, in the experiment 3 performed at industrial scale, using an intermittent day-night ozone cycle (1.6 mg kg^{-1} ozone-enriched atmosphere 12 h at night), decay incidence was not detected, as previously observed in the experiment 1.

3.3. Effects of ozone on fruit quality at industrial facility

3.3.1. Oleocellosis incidence

In experiment 1 three citrus varieties (Fortune, Lanelate and Navelate) exhibited this disorder. Oleocellosis occurred in both control citrus (citrus stored under air atmosphere in the cold chamber) and ozone-treated citrus. Oranges were more sensitive to this disorder than mandarins. When using 60 mg kg^{-1} ozone-enriched atmosphere instead of solely air, oleocellosis development increased in Lanelate and Navelate oranges, while the opposite effect was found in Fortune mandarins (Fig. 6). Similar results were found in experiments 2 and 3 (data not shown). However, the effect of ozone on oleocellosis in these experiments was not as clear as in experiment 1, since no significant differences (p<0.05) were found between control and ozone-treated citrus in most cases.

3.3.2. Colour

In experiment 1, the colour index did not show significant differences (p<0.05) between fruit treated and not treated with ozone (Fig. S1 and S2). During storage at 5 °C the colour of citrus barely changed. A general increase in the mean values was observed in the subsequent shelf life at 20 ºC, without noticeable differences between treatments. In experiment 2 (using also 60 mg kg^{-1} ozone continuously during storage at 5 °C), the colour index was significantly higher in fruit stored under air over both cold storage (Fig. S3) and shelf life at 20 ºC (Fig. S4). In experiment 3, applying 1.6 mg kg^{-1} ozone-enriched atmosphere in a day-night cycle, ozone-treated oranges showed a slower

development in the colouring process over the first 14 d of cold storage and subsequent shelf life at 20 °C. This effect was not observed from the third storage week (Table 2).

3.3.3. Firmness losses

In experiment 1 (cold storage under 60 mg kg^{-1} ozone-enriched atmosphere), ozone had a significant effect on the firmness of citrus fruit until the first week of shelf life (Fig. 7 and S5). Ozone-treated citrus lost firmness slower than untreated citrus. However, when placing the fruit in the chamber at 20°C, the differences in firmness start to diminish from the first week of shelf life, being the difference of percentage of firmness losses between treated and untreated citrus almost nil at the end of shelf life. In experiment 2, oranges treated with ozone also preserved firmness better than those stored under air during storage at 5°C. This effect was more noticeable in the variety Valencia (Fig. S6).

Navelate oranges treated with ozone showed minor firmness losses within the 15 d of shelf life at 20 ºC (Fig. S7). The fruit of variety Valencia treated with ozone showed lower firmness losses until day 10 of shelf life at 20 °C. From this time differences were not observed with respect to oranges preserved in air during cold storage. In experiment 3, in which an intermittent ozone-enriched atmosphere was applied during storage at 5°C, results showed that oranges preserved under 1.6 mg kg^{-1} ozone were firmer than fruit preserved under air during the first 19 d. Afterwards, firmness losses were equalized in both groups (Table 2). This effect of ozone on firmness disappeared in the subsequent shelf life at 20 ºC and values of firmness losses of untreated fruit and those that had previously been treated with ozone (Table 3) were alike.

3.3.4. Weight losses

Weight losses are of major importance from economic and qualitative terms. The economic factor is affected because citrus are sold by weight. Furthermore, the

transpiration of the fruit and consequent weight losses affects their quality. The presence of wrinkly citrus or simply fruit with lack of turgidity can lead to rejection by customers. Weight losses of citrus cold-stored under 60 mg kg^{-1} ozone-enriched atmosphere were always lower than those of citrus stored under air at 5°C in experiments 1 (Fig. 8 and S8) and 2 (Fig. S9). Once out the cold chamber, both untreated and ozone-treated citrus began to lose weight in a similar way, maintaining the initial differences (those generated during cold storage) throughout shelf life (Fig. 8, S8 and S10). The application of 1.6 mg kg^{-1} ozone-enriched atmosphere 12 h a day similarly led to a significant decrease of weight losses from the 14 d of cold storage, but during the subsequent shelf life the differences were increasing (Table 2).

3.3.5. Juice content, pH, vitamin C, soluble solids and titratable acidity

There were not significant differences between treated and control citrus fruit in relation to their juice content, pH, soluble solids and titratable acidity. The application of 60 mg kg^{-1} ozone-enriched atmosphere during cold storage did not exert any influence on juice content (Fig. S11-S13). Neither was detected over shelf life (Fig. S11, S12 and S14). However, some noticeable differences were found in experiment 2 during shelf life (Fig. S14), but without any trend, so conclusions about the effect of ozone on juice content could not be extracted. Similarly to experiments 1 and 2, juice content did not seem to be affected in the subsequent shelf life after the application of intermittent 1.6 mg kg^{-1} ozone during cold storage (Table 2).

Ozone treatment did not induce any effect on pH of juices in any of the 3 experiments (Fig. S15-S17 and Table 3). The same fact was observed in titratable acidity and vitamin C (Table 3, data not shown for experiments 1 and 2).

In regard to soluble solids, the application of 60 mg kg^{-1} ozone during cold storage did not exert any influence on soluble solids in experiments 1 and 2 (Fig. S18-S21). By contrast, significant differences in concentration of soluble solids between control and ozone-treated citrus were found in experiment 3 (Table 3), being the first ones those with higher concentrations of soluble solids during storage at 5 °C. In the subsequent period of shelf life, these significant differences were observed as well.

3.4. Sensory analysis of the citrus juice

Sensory analysis did not show significant differences in the ranking order (Friedman test) among juice samples (data not shown). When asked, panellists did not find any negative effect of ozone on fruit quality, developing neither atypical flavour nor off-flavour. Similarly, juices from fruit stored up to 4 weeks under ozone-enriched atmosphere did not develop any atypical flavour or off-flavour.

4. Discussion

4.1. Assays at laboratory scale

4.1.1. Effects of ozone concentration on *in vitro* fungal growth

Palou et al. (2001) observed that the *in vitro* radial growth of *P. italicum* during 5-day incubation at 20 ºC was significantly reduced by a previous 0.30 ± 0.05 mg kg^{-1} ozone exposure at 5 ºC for 4 d. Interestingly, this exposure to ozone did not inhibit *in vitro P. digitatum* growth over the subsequent 5-day incubation at 20 ºC. However, Karaka (2010) observed that gaseous ozone was able to prevent microbial activity even at low doses owing to its high oxidizing potential. According to our results, storage at 5 ºC under an atmosphere with an ozone concentration of 1.6 mg kg^{-1} simulating a day-night cycle (12 h on, 12 h off) was the most suitable scheme for inhibiting the *in vitro* growth

of the two studied fungi. Its increased efficiency could be explained by the more difficult adaptation of fungi to the oxidizing environment caused by ozone. Under continuous ozone atmosphere, fungi have to adapt solely to the new atmosphere. Therefore, these conditions were selected for further laboratory and industrial assays.

4.1.2 Effects of ozone concentration on in vivo *P. italicum* growth

As mentioned earlier, storage under ozone atmosphere (1.6 mg kg^{-1} 12 h d^{-1}) increased about 3 times the lag phase duration of the fungus in wound-inoculated oranges and also reduced its exponential growth. This is in agreement with other authors' findings. Harding (1968) observed that the growth of *P. italicum* and *P. digitatum* was inhibited by using continuously 1 mg kg^{-1} ozone during storage. However, this author reported that once the fruit were taken out the ozonified chamber both microorganisms rapidly started growing. Similarly, Palou et al. (2001) found that the incidence of *P. italicum* and *P. digitatum* on wound-inoculated citrus during shelf life was delayed by previous continuous exposure to 0.10 ± 0.05 mg kg^{-1} ozone at 10 ºC for 2 weeks in an export container. However, in spite of the fact that infections developed slower under ozone than under air, these authors found that decay incidence at the end of the storage period was not reduced. On the contrary, Di Renzo et al. (2005) reported that intermittent gaseous ozone at 0.25 mg kg^{-1} did not reduce *Penicillium* mold incidence in non-washed oranges, which is in contrast with most of researches. Regarding other fruit, Liew and Prange (1994) slowed down to 50% the growing of *Botrytis cinerea* and *Sclerotinia sclerotiorum* inoculated on carrots, which were stored at 2, 8 and 16 ºC, using a much higher ozone concentration (60 mg kg^{-1}). As afore mentioned, the application of ozone in an intermittently way leads to improved results and allows the use of lower ozone concentrations.

The results related to the delay of the development of the fungus on oranges can be considered of high interest to the industry, since oranges could be stored in chambers for greater periods of time, extending their shelf lives and thus delaying the appearance of rotten oranges, with the consequent reduction in losses during the post-harvest period. Moreover, the fact that oranges preserved under ozone-enriched atmosphere did not develop sporulation has a special significance, since the use of intermittent ozone would hinder the spread of the fungus to healthy fruit. Healthy oranges in close contact with already-infected oranges through the mycelium could be infected, but the presence of spores suspended in the atmosphere of the chamber would be restricted. What is more, the use of an intermittent day-night ozone cycle would facilitate the work of operators, who could handle the oranges inside the camera during the 12 h without ozone treatment, thus avoiding health hazards due to ozone exposure. This is of major importance for industrial application because a threshold of 0.1 mg kg^{-1} ozone for continuous exposure in the workplace environment during an eight-hour day/40-h work week period and 0.3 mg kg^{-1} ozone for a 15-min period have been stipulated by the Federal Occupational Safety and Health Administration (OSHA) of the United States and the Health and Safety Executive (HSE) of the United Kingdom (Karaca and Velioglu, 2007).

4.2. Decay incidence in ozone trials at industrial facility

The results agree with those found by Harding (1968), who observed that the application of 1 mg kg^{-1} ozone to orange and lemons at 14 °C slightly reduced the decay incidence and sporulation, and with those of Metzger et al. (2007) under semi-commercial conditions, who observed that the percentage of rotten fruit was significantly reduced by 7% after 35-day storage under 0.18–0.20 mg kg^{-1} ozone-

enriched atmosphere. It is worth to note that ozone must penetrate into boxes or containers where fruit are stored. Similarly to us, Harding (1968) obtained reduction in citrus decay incidence with open boxes, whereas Palou et al. (2001) reported that ozone penetration into cartons with small vents was not enough to reduce citrus decay. In a subsequent work, these authors packed oranges in California standard citrus cartons, naked or bagged (in polyethylene bags) in vented returnable plastic containers, or bagged in fibreboard Master cartons and subjected them to 0.72 mg kg^{-1} ozone exposure for 14 d (Palou et al., 2003), and concluded that the practical use of ozone gas exposure during citrus cold storage was limited to highly vented packages or open-top containers. Ozone will probably fail in inactivating microorganisms located deep inside citrus, because it has a low penetration power. However, microbial infections, such as green and blue mold, generally start from citrus surface (Karaca, 2010), so ozone may be effective against them.

With regard to other fruit, Norton et al. (1965) found that decay incidence was reduced by storing cranberries at 4 °C for 8 weeks under 0.27 mg kg^{-1} ozone atmosphere. However, storage at 16 °C for 5 months using 0.06 mg kg^{-1} ozone led to the opposite effect. Barth et al (1995) also reported that storage of blackberries under 0.1 or 0.3 mg kg^{-1} ozone atmosphere for 12 d at 2 °C was effective in preventing losses due to rotting. Sarig et al. (1996) observed that a short exposure to ozone (20 minutes) reduced the incidence decay in table grapes during cold storage and subsequent shelf life. According to the authors, ozone not only had a sterilizing effect but also induced resistance against the development of fruit rot. Cayuela et al. (2009) concluded that 2 mg kg^{-1} ozone treatment for 72 d considerably reduced decay of cold stored grapes compared to those kept in air. As above mentioned, decay incidence at industrial scale was solely detected in experiment 2. This was a hindrance for our research, because the comparison

between the effects of continuous and intermittent application of ozone would be very valuable. In this sense, Cayuela et al. (2009) found that continuous 2 mg kg^{-1} ozone treatment was more effective than intermittent (12 h d^{-1}) 2 mg kg^{-1} ozone treatment for controlling grape postharvest losses. Therefore, the effect of ozone on this important parameter could not be accurately determined at industrial scale. Anyway, the results from decay incidence showed that the effectiveness of exposure to ozone as pretreatment for improving citrus shelf life is superior to that of heat treatments both at laboratory (water dipping) and industrial scale (heat water showers system) reported in a previous work (García et al., 2016).

4.3. Effects of ozone on fruit quality at industrial facility

Oleocellosis is a physiological disorder provoked by the rupture of peel oil glands, releasing their content which is phytotoxic to pericarp cells (Scherrer-Montero et al., 2012). Although this disorder is mainly associated to mechanical injuries (causing thus high postharvest losses), ozone, a strong oxidant, could provoke oleocellosis in citrus because it can alter the permeability of the membrane of the oil glands. Therefore, we assessed whether ozone was also responsible for citrus oleocellosis during cold storage and shelf life. As mentioned earlier, we found similar results in the 3 experiments, but only in one of them (experiment 1) there were significant differences (p<0.05) in oleocellosis incidence between control and ozone-treated citrus. The increase in the incidence of oleocellosis induced by the presence of ozone in the storage atmosphere observed in the oranges Lanelate and Navelate could be foreseeable. However, the fact that the variety of Fortune exhibited the opposite answer evidences that the relationship between this disorder and the action of ozone is more complicated and varies according

to the variety. In any case, considering all the experiments, it can be concluded that the effect of ozone on citrus oleocellosis is quite limited.

The results obtained about colour index could be probably due to the fact that ozone partially inhibits the synthesis of carotenoids, components responsible for the final colour of oranges. Therefore, ozone treatment should preferably be applied to oranges that have reached an acceptable level of pigmentation.

From the results of the 3 experiments, it can be concluded that ozone has a positive effect in preserving citrus firmness during cold storage. Greater firmness in citrus is a reliable indicator that such fruit will be more resistant to fungal infection (García et al., 2016). In this sense, ozone treatment can be regarded as beneficial to citrus quality.

Ozone likely reduced the permeability of citrus, decreasing thus their ability to transpire. This effect does not disappear when the fruit are taken to a normal atmosphere (air). Therefore, the effect of ozone on water permeability of citrus accounted for the differences in weight losses registered: lower weight losses were registered during cold storage under ozone atmosphere, when compared with cold storage under air, and once oranges were taken out the cold chamber, both untreated and ozone-treated citrus began to lose weight in a similar way, maintaining the weight losses difference observed during cold storage over the subsequent shelf life. This effect of ozone on weight losses agrees with Di Renzo et al.'s findings (2005), who reported that intermittent exposure of oranges to 0.25 mg kg^{-1} gaseous ozone reduced their ageing and weight losses more than when oranges were exposed solely to air. Our results are opposite to those of Norton et al. (1965), who observed that cranberries preserved at 5°C under 0.27 mg kg^{-1} ozone-enriched atmosphere suffered an increase in weight losses compared to cranberries stored under air at the same temperature. Similarly, 2 mg kg^{-1} ozone treated

grapes showed significantly higher weight losses than fruit kept under air at 5 ºC (Cayuela et al., 2009).

Similarly to the results we obtained in a previous work about postharvest heat treatment (García et al., 2016), juice content, pH, vitamin C, soluble solids and titratable acidity of citrus were barely modified by ozone postharvest treatment, which means that exposure to ozone was not detrimental to these citrus quality parameters. This is consistent with the results of Carbone and Mencarelli (2015) on the exposition of grapes to ozone gas in air atmosphere for 12 h at 10 ºC, who concluded that the use of ozone in air or nitrogen does not alter the fruit quality attributes. The results on total soluble solids found in the experiment 3 (higher concentration in control fruit than in ozone-treated citrus) are in contrast with Kute et al.'s findings on ozone postharvest of strawberries (Kute et al., 1995), who found that strawberries subjected to 0.3 or 0.7 ozone at 2 °C showed an increase in soluble solids content during the first week of storage, reaching much higher values than strawberries preserved under air.

4.4. Sensory quality

The absence of effect on the sensory quality due to the use of ozone could be regarded as a positive result since other fruit exposed to ozone in air atmosphere, such as grapes, got lower scores than control fruit in the sensory evaluation tests (Cayuela et al., 2009). Nevertheless, in case of adverse effects derived from ozone treatments, these could disappear over time. Thus, Nadas et al. (2003) reported that detrimental effects on strawberry aroma found after cold storage (2 ºC) under 1.5 mg kg^{-1} ozone-enriched atmosphere disappeared after 2 d of shelf life.

5. Conclusions

Storage under ozone atmosphere markedly inhibited the *in vitro* growth of *P. digitatum* and *P. italicum*. This effect was enhanced when applying 1.6 mg kg^{-1} ozone simulating a day-night cycle. In *in vivo* experiments, the lag phase of growth of *P. italicum* in oranges stored under intermittent 1.6 mg kg^{-1} ozone atmosphere was 3 times higher (15 d) than that of oranges stored under air (5 d). The subsequent exponential growth stage of *P. italicum* was also quicker in control oranges. At the industrial facility, the exposition of citrus fruit to an ozone-enriched atmosphere during cold storage reduced decay incidence. However, oleocellosis incidence increased in Lanelate and Navelate oranges, while the opposite effect was found in Fortune mandarins. The presence of ozone in the storage atmosphere delayed development of red colour on fruit during shelf life and limited weight and firmness losses. The use of an ozone-enriched atmosphere in the cold storage chamber did not affect quality parameters such as juice content, soluble solids content, pH, vitamin C and titratable acidity values of citrus fruit during shelf life. All in all, results may be regarded as positive and illustrate the potential of gaseous ozone treatment as an alternative to postharvest fungicides to control citrus green and blue molds. Finally, the application of intermittent ozone simulating a day-night cycle is strongly recommended not only because the obtained results, but also because workers would not be exposed to ozone during day shift.

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Figure captions

Fig. 1. Evolution of *Penicillium digitatum* growth in PDA at 5 ºC under air or different ozone-enriched atmospheres: Air (black squares); 0.6 mg kg^{-1} O_3 (grey triangles); 1.6 mg kg^{-1} O_3 (black triangles); 1.6 mg kg^{-1} O_3 intermittent 12 h d^{-1} (grey diamond) and 60 mg kg^{-1} O_3 (black circles). Data are the mean value ± SD of 40 replicates.

Fig. 2. Evolution of *Penicillium italicum* growth in PDA at 5 ºC under air or different ozone-enriched atmospheres: Air (black squares); 0.6 mg kg^{-1} O_3 (grey triangles); 1.6 mg kg^{-1} O_3 (black triangles); 1.6 mg kg^{-1} O_3 intermittent 12 h d^{-1} (grey diamond) and 60 mg kg^{-1} O_3 (black circles). Data are the mean value ± SD of 40 replicates.

Fig. 3. Decay incidence of Valencia oranges inoculated with *P. italicum* over storage at 5 ºC under air (open circle) and under intermittently 1.6 mg kg^{-1} ozone atmosphere 12 h d^{-1} (filled circle), respectively. Data are the mean value ± SD of 4 replicates of 20 oranges.

Fig. 4. Effect of cold storage at 5 º C under air (filled symbols) and 60 mg kg^{-1} ozone-enriched atmosphere (open symbols) on decay incidence of Lanelate (squares) and Valencia (triangles) oranges in experiment 2. Data are the mean value ± SD of 4 replicates.

Fig. 5. Decay incidence of Lanelate (squares) and Valencia (triangles) oranges over shelf life at 20 ºC, after a) 7, b) 14, c) 21 and d) 28 d of cold storage at 5 ºC under 60 mg kg^{-1} ozone-enriched atmosphere (open symbols) and air (filled symbols) in experiment 2. Data are the mean value of 4 replicates ± SD.

Fig. 6. Evolution of oleocellosis incidence on Fortune mandarins (squares) and Lanelate (triangles) and Navelate (circles) oranges over the whole postharvest period in experiment 1. Open symbols: cold storage under 60 mg kg^{-1} ozone; filled symbols: cold storage under air. Data are the mean value ± SD of 4 replicates.

Fig. 7. Evolution of firmness losses of Fortune (squares) and Ortanique (triangles) mandarins over the whole postharvest period in experiment 1. Open symbols: cold storage under 60 mg kg^{-1} ozone; filled symbols: cold storage under air. Data are the mean value ± SD of 20 replicates.

Fig. 8. Changes of weight losses of Lanelate (triangles), Navelate (circles) and Salustiana (diamonds) oranges over the whole postharvest period in experiment 1. Open symbols: cold storage under 60 mg kg^{-1} ozone; filled symbols: cold storage under air. Data are the mean value of 10 replicates.

Table 1.

Ozone experiments with citrus at industrial scale

Experiment	Orange varieties	Mandarin varieties	Storage atmosphere	Storage time (d)	Shelf life (d)
1	Salustiana, Lanelate and Navelate	Fortune and Ortanique	60 mg kg^{-1} O_3	28	14
2	Lanelate and Valencia	None	60 mg kg^{-1} O_3	7, 14, 21 and 28	15
3	Valencia	None	1.6 mg kg^{-1} O_3 (12 h d^{-1})	7, 14, 21 and 28	10

Table 2.

Changes of quality parameters of Valencia oranges in experiment 3 during shelf-life after storage at 5°C for different times under air and under 1.6 mg kg^{-1} ozone-enriched atmosphere for 12 h a day.

Storage (d)	Shelf Life (d)	Colour (1000a*L*$^{-1}$b*$^{-1}$)		Firmness (N)		Weight losses (%)		Juice (mL kg^{-1})	
		Air	Ozone	Air	Ozone	Air	Ozone	Air	Ozone
0	**Raw orange**	3.4±0.3		30.0±0.2		0		424±35	
7	**0**	3.9±0.2a	3.4±0.3b	28.5±0.3b	29.3±0.3a	1.8±0.2	1.6±0.2	501±43	431±39
	4	3.9±0.2a	3.5±0.2b	24.8±0.4b	25.9±0.4a	7.9±0.3a	7.1±0.3b	432±37	465±40
	10	4.2±0.3a	3.7±0.3b	21.6±0.4	21.9±0.5	12.4±0.5a	11.0±0.4b	481±42	453±39
14	**0**	4.0±0.2a	3.7±0.2b	28.4±0.3b	29.0±0.3a	2.7±0.2a	2.3±0.2b	457±45	444±38
	4	4.2±0.2a	3.8±0.2b	25.5±0.3	25.6±0.4	8.3±0.3a	7.5±0.4b	506±47	471±40
	10	4.7±0.4a	3.7±0.2b	22.4±0.3	22.6±0.3	12.5±0.4a	11.3±0.5b	440±38	455±36
21	**0**	4.2±0.3	4,2±0.2	27.4±0.3b	28.5±0.4a	3.5±0.2a	3.0±0.2b	430±34	421±35
	4	4.3±0.3	4.2±0.4	24.6±0.3	25.2±0.3	8.9±0.5	7.7±0.5	452±41	459±43
	10	4.5±0.3	4.4±0.4	21.9±0.4	22.2±0.4	13.0±0.6	11.8±0.6	453±45	416±35
28	**0**	4.2±0.3	4.2±0.3	27.0±0.3	27.2±0.3	5.0±0.2a	4.5±0.2b	435±42	417±37
	4	4.5±0.4	4.3±0.3	25.6±0.4	26.2±0.4	9.9±0.5a	8.3±0.6b	423±32	432±41
	10	4.4±0.4	4.3±0.4	21.9±0.4	22.4±0.5	13.4±0.6a	12.1±0.6b	452±39	461±40

Different letters in the same day indicate significant differences according to ANOVA ($p \leq 0.05$) and the absence of letter means no significant effect due to the treatment. Results are the mean values ± SD of 20 replicates.

Table 3. Changes of quality parameters of Valencia orange juices in experiment 3 during shelf-life after storage at 5°C for different times under air and under 1.6 mg kg^{-1} ozone-enriched atmosphere for 12 h a day.

Storage (d)	Shelf Life (d)	Acidity (%)		pH		Soluble solids (%)		Vitamin C (g L^{-1})	
		Air	Ozone	Air	Ozone	Air	Ozone	Air	Ozone
0	Raw orange	0.9±0.2		3.8±0.4		10.2±0.3		0.40±0.04	
7	0	0.9±0.2	0.9±0.3	3.6±0.3	3.5±0.3	11.4±0.2a	10.8±0.2b	0.37±0.03	0.37±0.04
	4	1.0±0.3	0.8±0.3	3.6±0.4	3.8±0.4	11.6±0.2a	10.7±0.3b	0.42±0.04	0.41±0.04
	10	0.9±0.3	0.8±0.3	3.7±0.3	3.7±0.4	11.3±0.3	11.2±0.4	0.39±0.04	0.36±0.03
14	0	0.9±0.2	0.8±0.3	3.6±0.3	3.5±0.3	11.5±0.3a	10.7±0.2b	0.37±0.04	0.36±0.04
	4	1.1±0.3	1,2±0.1	3.6±0.2	3.7±0.3	11.5±0.4a	10.6±0.3b	0.42±0.05	0.34±0.06
	10	0.8±0.2	0.8±0.2	3.7±0.3	3.7±0.2	10.9±0.4	11.3±0.4	0.40±0.04	0.39±0.05
21	0	0.9±0.2	0.8±0.2	3.7±0.4	3.7±0.3	11.6±0.3a	10.5±0.2b	0.38±0.04	0.37±0.04
	4	0.9±0.2	0.8±0.1	3.7±0.3	3.8±0.4	11.3±0.4a	10.6±0.3b	0.39±0.03	0.39±0.04
	10	0.8±0.2	0.7±0.2	3.9±0.3	4.0±0.4	11.6±0.4a	10.0±0.5b	0.33±0.05	0.33±0.04
28	0	1.0±0.2	0.8±0.2	3.7±0.3	3.8±0.3	11.9±0.4a	10.7±0.4b	0.40±0.05	0.39±0.04
	4	0.9±0.2	0.8±0.1	3.8±0.3	3.9±0.3	9.9±0.5a	8.3±0.6b	0.43±0.05	0.41±0.04
	10	1.0±0.3	0.9±0.2	3.8±0.3	3.8±0.4	13.4±0.6a	12.1±0.6b	0.36±0.04	0.40±0.05

Different letters in the same day indicate significant differences according to ANOVA ($p \leq 0.05$) and the absence of letter means no significant effect due to the treatment. Results are the mean values ± SD of 20 replicates.

Figure 1

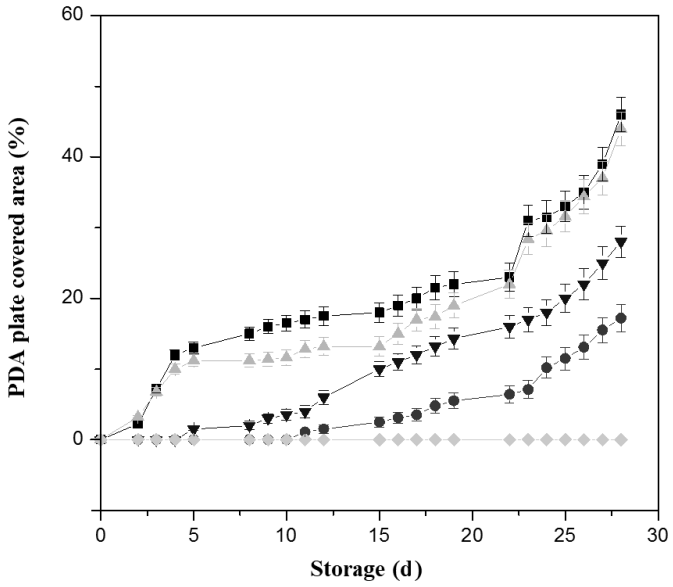


Figure 2

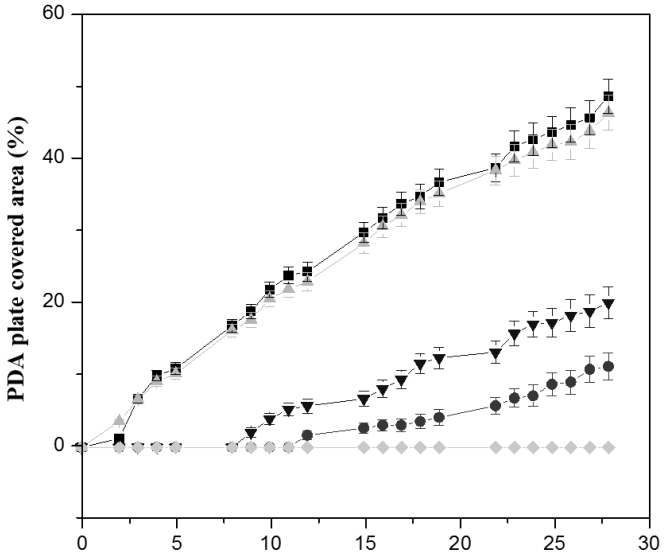


Figure 3

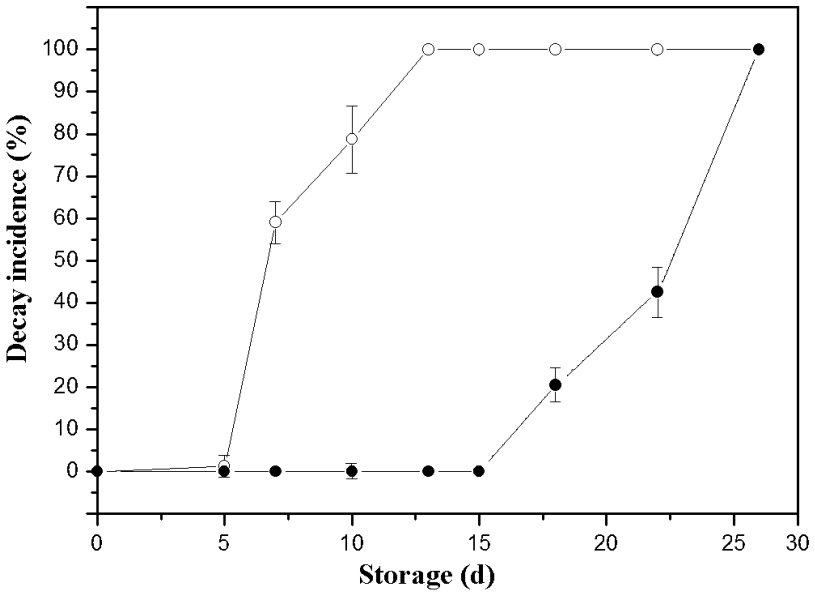

Decay incidence (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Storage (d)

Figure 4

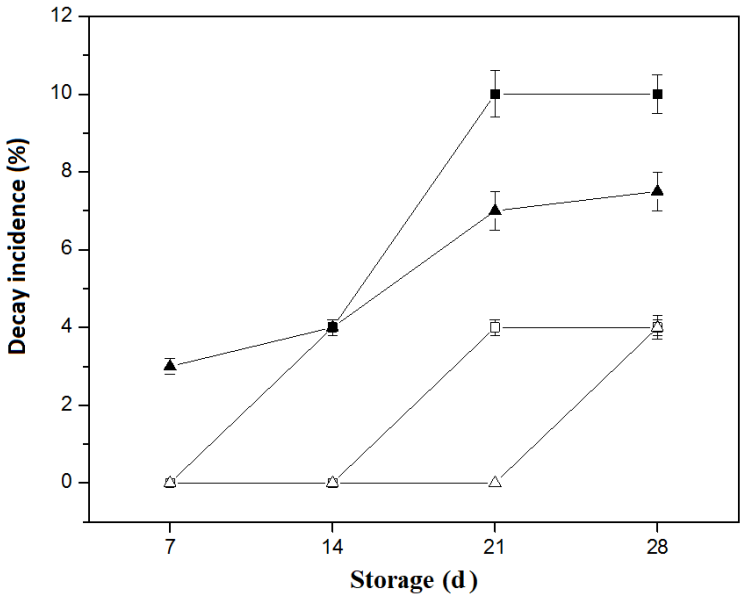


Figure 5

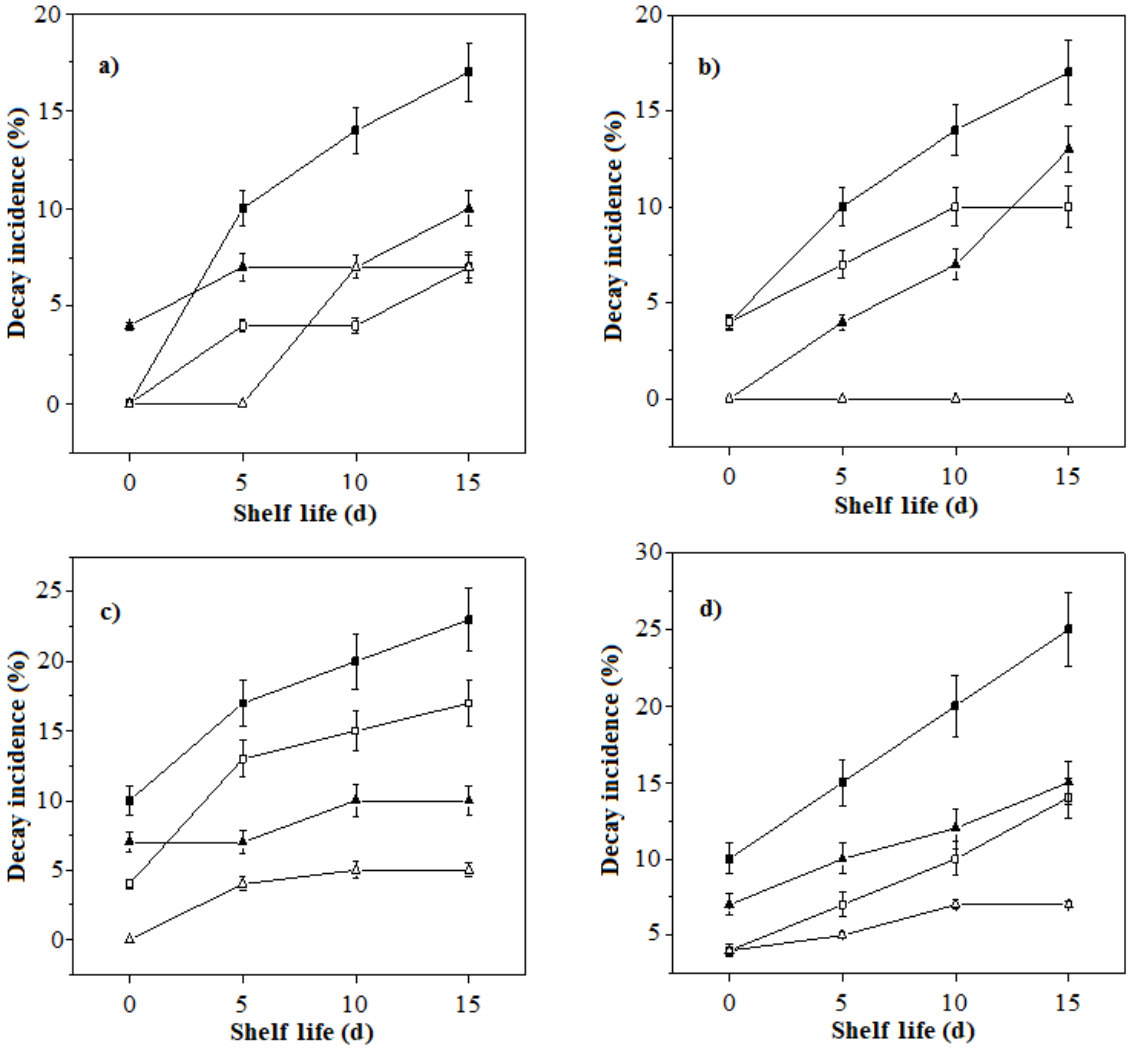


Figure 6

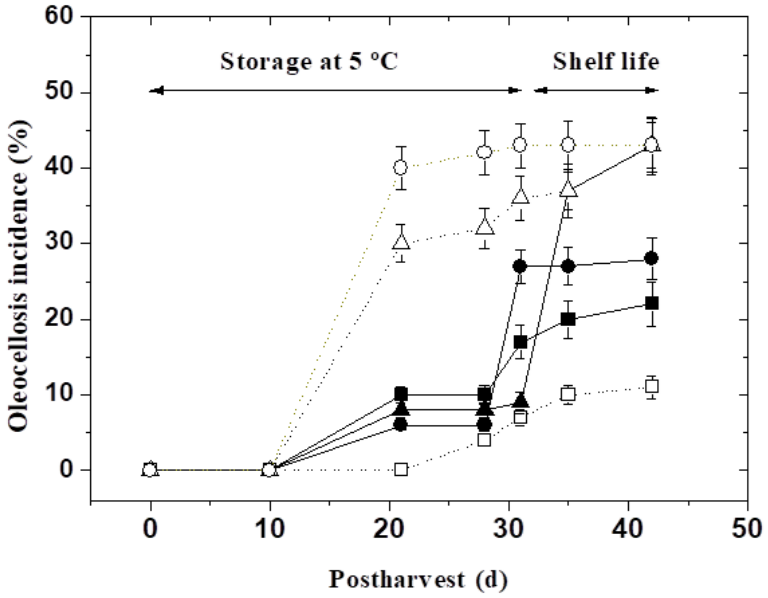

Storage at 5 °C
Shelf life
Oleocellosis incidence (%)
Postharvest (d)
0
10
20
30
40
50
60

Figure 7

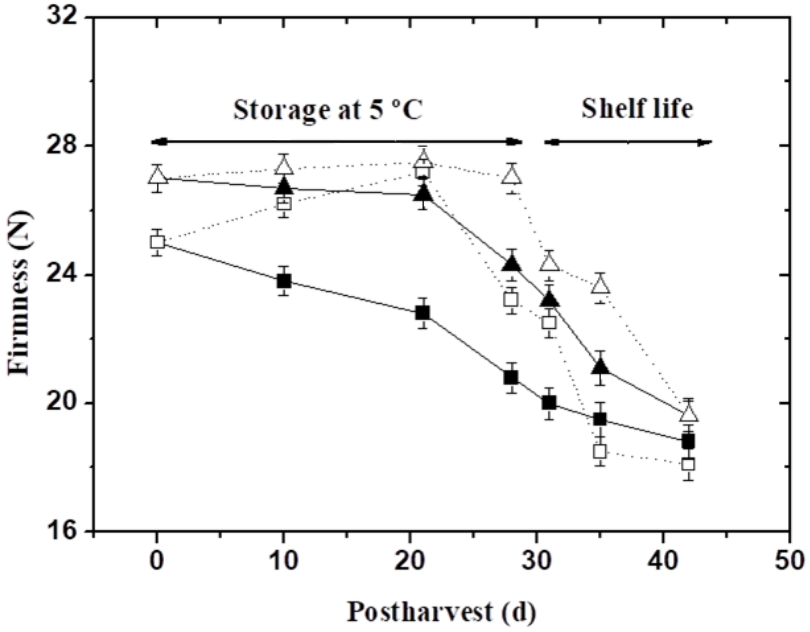
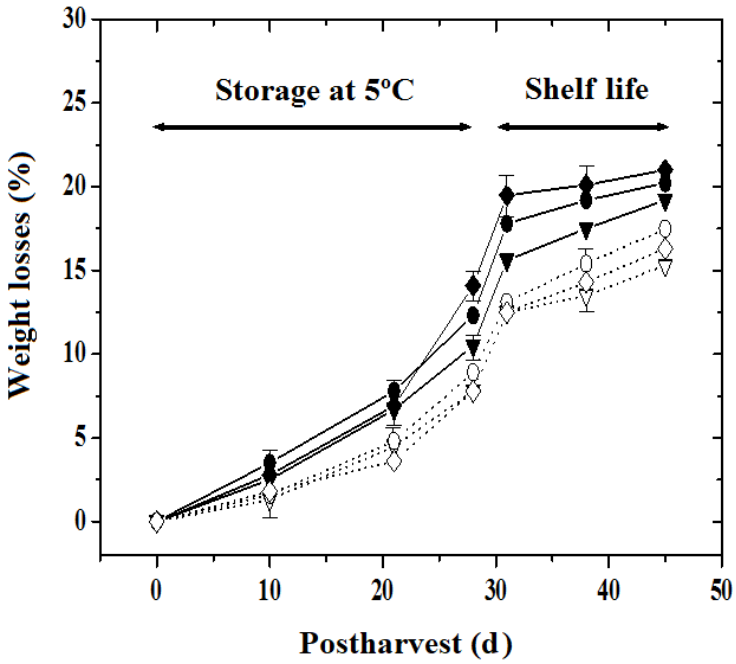

Storage at 5 °C
Shelf life
Firmness (N)
Postharvest (d)
32
28
24
20
16
0
10
20
30
40
50

Figure 8


Storage at 5°C
Shelf life
Weight losses (%)
Postharvest (d)
0
5
10
15
20
25
30
0
10
20
30
40
50