Short-Term Exposure to High CO₂ and O₂ Atmospheres to Inhibit Postharvest Gray Mold of Pomegranate Fruit

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Abstract

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The effect of short-term exposure to high CO₂ or O₂ atmospheres, alone or in combination with heat (35°C), for the control of postharvest gray mold was evaluated on 'Mollar de Elche' pomegranate fruit artificially inoculated with *Botrytis cinerea* and stored at 20 or 5°C. Exposure to high CO₂ for 48 h at 20°C effectively reduced gray mold on pomegranate fruit incubated at 20°C for 5 days in a concentration-based manner. Furthermore, gaseous treatments with partial pressures of 95 kPa CO₂ or 30 kPa O₂ + 70 kPa CO₂ for 48 h significantly reduced gray mold incidence and severity on fruit regardless of storage temperature. Moreover,

for fruit receiving gaseous treatments at 20°C then cold stored for 12 weeks, there were no apparent negative effects of the treatments on their quality (weight loss, skin color, maturity index, pH, and sensory quality). In general, compared with those applied at 20°C, treatments at 35°C did not improve gray mold inhibition and adversely affected some quality parameters. In conclusion, 48-h exposures to specific atmospheres at 20°C may be a suitable treatment to extend pomegranate storage life and could be part of integrated control programs to control postharvest decay.

Pomegranate (*Punica granatum* L.) is one of the oldest fruit known to mankind, with traditional uses for food, medicine, and tanning leather (Verma et al. 2010). It is native to Persia (central Asia, currently Iran), from which it spread to the rest of the world, and today it is grown in many different geographical regions, including the Mediterranean basin, Asia, and California (Holland et al. 2009). Spain is the main producer and exporter of pomegranate fruit in the European Union (EU), with a production in 2012 of 36,000 tons, of which 98% were exported, mainly to EU markets (MAGRAMA 2012). The importance of this crop in Spain is increasing following the recent global trend of escalation of pomegranate demand due, in part, to its perceived healthful properties (Díaz-Rubio et al. 2015; Lansky and Newman 2007). Because of this increasing demand, there is an interest from the pomegranate industry in extending the storage life of the fruit into out-season markets. The most widely grown cultivar in Spain, 'Mollar de Elche', is commonly harvested from late September to mid-November, and it would be desirable to prolong their commercial storage until December, when prices are higher in the EU markets.

Factors limiting the storability of pomegranate include weight loss, fungal decays, and chilling injury. Cold storage at high relative humidity (95% RH) is an effective technology to reduce weight loss in pomegranate fruit. However, temperatures between -3°C (pomegranate freezing point) and 5°C can cause chilling injury, which is characterized by brown discoloration of the skin, surface pitting, pale color of the arils (edible part of the fruit), and increased susceptibility to decay (Kader et al. 1984). Storage at temperatures above 5°C to avoid chilling injury increases the risk of losses by decay fungi (Ben-Arie and Or 1986; Elyatem and Kader 1984). For example, for fruit with a high level of quiescent fungal infections of *Botrytis cinerea* Pers. at the time of harvest and stored between 5 and

7.5°C, the storage life was reduced up to 8 weeks compared with fruit with a low level of quiescent infection (Hess-Pierce and Kader 2003). Typically, gray mold caused by *B. cinerea* is among the most important postharvest diseases of pomegranate worldwide (Pala et al. 2009; Tedford et al. 2005; Thomidis 2014). In California, during the 1999 to 2002 growing seasons, a period when no postharvest fungicides were registered, this disease affected up to 30% of harvested pomegranate fruit (Tedford et al. 2005). In Spain, *B. cinerea* was the most frequent pathogen causing latent infections on Mollar de Elche pomegranate fruit and, together with *Penicillium* spp., it was also the main cause of postharvest wound infections (Palou et al. 2013). Conidia of *B. cinerea* present on the rind of mature fruit can infect through injuries or cracks but postharvest decay originating from quiescent infection established before harvest is usually more important. *B. cinerea* conidia are produced in large amounts on many different organic sources and are dispersed through dry air currents. Conidia infect flower tissues if there is free water available. The pathogen can then remain quiescent and only after harvest resume its growth and spread from the crown to the body of the fruit, causing typical gray mold symptoms (Adaskaveg and Michailides 2013). In addition, *B. cinerea* is able to infect stored pomegranate fruit by mycelial spread from infected fruit to adjacent healthy fruit, causing nests of decay (Palou et al. 2007). The application of fungicides reduces postharvest decay losses and, to date, is a key factor to ensure pomegranate industry profitability in certain areas such as California (Tedford et al. 2005).

Because there are problems related to the use of fungicides, such as a potential to develop resistant strains of pathogens and the increasing public concern for food safety and chemical residues, we have been interested in alternative, nonchemical postharvest technologies that extend pomegranate commercial life while maintaining fruit quality. These technologies include fruit conditioning and curing (Artés et al. 2000; Mulas and Schirra 2007), modified atmospheres (MA) (Caleb et al. 2012; Selcuk and Erkan 2014), and controlled atmospheres (CA) (Artés et al. 1996; Hess-Pierce and Kader 2003). CA is considered the most efficient technology, extending postharvest life of pomegranate by delaying fruit senescence and inhibiting the growth of microorganisms causing decay (Ben-Arie and Or 1986). Hess-Pierce and Kader (2003) found that it was possible to store pomegranate fruit ('Wonderful') at 7.5°C in 5 kPa O₂ + 15 kPa CO₂ (atmosphere is approximately 5% O₂ + 15% CO₂) for up to 5 months, as long as the number of quiescent fungal infections at

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the time of harvest was low. On this cultivar, dip treatments in aqueous solutions of common food preservatives such as potassium sorbate or sodium carbonates in combination with a CA of 5 kPa O₂ + 15 kPa CO₂ also improved its postharvest quality (Palou et al. 2007). For Mollar de Elche pomegranate, Artés et al. (1996) recommended storage at 5°C in a CA of 5 kPa O₂ + 0.5 kPa CO₂ to prolong its storage life for up to 2 months while reducing decay and maintaining fruit quality. Long-term CA storage, however, is an expensive technology not easily applicable by many growers and difficult to maintain during product transportation. Short-term exposure to high CO₂ or O₂ can also ameliorate postharvest diseases of fresh fruit. For example, postharvest treatment with 30 kPa CO₂ for 24 h in avocado delayed the development of anthracnose symptoms caused by *Colletotrichum gloeosporioides* (Prusky et al. 1991). Similarly, in previous research (Montesinos-Herrero et al. 2012a), we found that exposure of artificially inoculated citrus fruit to 15 kPa CO₂ for 24 h or 30 kPa O₂ for 48 h at 33°C (low curing temperature) significantly inhibited green and blue molds caused by *Penicillium* spp. Another short-term alternative postharvest technology used to reduce fungal decay of fruit is curing. This heat treatment consists of keeping the fruit for 12 to 94 h at 30 to 46°C and 90 to 98% RH and has been reported as an effective method to control decay of fresh fruit, especially in citrus (Schirra et al. 2000).

We hypothesize that short exposures to atmospheres able to inhibit fungal growth, alone or in combination with curing temperatures, could control gray mold development on pomegranate fruit after harvest. In the present work, we evaluated short-term exposure of Mollar de Elche pomegranate fruit artificially inoculated with *B. cinerea* to CO₂ or O₂ levels higher than those used in CA, combined or not with heat (35°C), as a method to control gray mold of pomegranate. Additionally, the effects of selected treatments on fruit quality were also assessed. Preliminary results from this study have been published (Montesinos-Herrero et al. 2012b).

Material and Methods

Fruit. For two seasons, Mollar de Elche pomegranate, commercially grown in the Elx area (Alicante Province, Spain), were harvested at commercial maturity and transported to the Pathology Laboratory at the Postharvest Technology Center of the Valencian Institute for Agricultural Research, where sound medium-sized fruit were selected, randomized, and stored at 5°C and 90% RH until used in the experiments. Prior to use, pomegranate fruit were surface disinfected by spraying to runoff with 98% ethanol.

Fungal inoculation. *B. cinerea* isolate GCA-3, obtained from a diseased pomegranate and selected for its pathogenicity on pomegranate, was maintained on potato dextrose agar in petri dishes at 25°C. Pomegranate fruit were injured on two opposite sides of the equatorial zone with a sterilized cork borer (5 mm in diameter) to leave a 2-mm-deep wound. Mycelial plugs excised with an identical cork borer from 14- to 21-day-old cultures of *B. cinerea* were inserted in each wound, with the growing aerial mycelia facing the interior of the fruit. The inoculated wound was then covered with adhesive tape to avoid desiccation of the tissues and favor infection. Fruit were kept at 20°C and 90% RH for 24 h; then, adhesive tapes were removed immediately before gaseous treatments were applied.

Gaseous treatments and storage conditions. Chambers used for gas exposure consisted of hermetically sealed, transparent polymethyl methacrylate cabinets (82 by 62 by 87 cm) fitted with outlet and inlet ports through which O₂ or CO₂ (Alphagaz; Air Liquide España S.A., Madrid) were injected until the desired concentrations of O₂ or CO₂ were achieved. The cabinets were also fitted with internal basal water trays that allowed RH as high as 90 ± 5%. Levels of O₂ and CO₂, temperature, and RH were continuously monitored by means of a computer-controlled system (Control-Tec; Tecnidex S.A., Paterna, València, Spain). The system injected O₂ or CO₂ from external pressurized cylinders until the desired target level was reached in each cabinet, with an oscillation of approximately ±2 kPa O₂ or CO₂. During the entire exposure period (48 or 24 h, depending on the experiment), the gas injection only resumed when the actual gas concentration in the cabinet was below the target value,

as detected through the periodic sampling performed automatically by the gas analyzer of the system.

In order to determine the effect of different O₂ or CO₂ concentrations and exposure times and temperatures on the development of gray mold on artificially inoculated pomegranate fruit, several experiments were conducted. First, pomegranate fruit (four replicates of five fruit each per treatment) were exposed for 48 h to an atmosphere containing air (control), CO₂ (15, 50, or 95 kPa), O₂ (30 or 45 kPa), or the combination of O₂ and CO₂ (40 kPa O₂ + 15 kPa CO₂) at 20°C. After exposure, fruit were arranged in plastic trays and incubated at 20°C and 90% RH for up to 11 days. In a second experiment, two gaseous atmospheres were selected to evaluate the combined effect of heat and high CO₂ and O₂ levels. Fruit were treated for 48 h at 20 or 35°C with air (control), 95 kPa CO₂, or the gaseous mixture 30 kPa O₂ + 70 kPa CO₂. Eight replicates of 5 pomegranate fruit (40 fruit in total) were used for each gaseous atmosphere and exposure temperature. After treatment, fruit were divided into two lots: the first was incubated at 20°C and 90% RH for 12 days and the second was kept at 5°C and 90% RH for up to 12 weeks. In a third experiment, in order to evaluate shorter exposure periods for selected gaseous treatments, pomegranate fruit (eight replicates of five fruit each per treatment) were treated for 24 h with air (control) or 95 kPa CO₂ at 20°C. After treatment, fruit were also divided into two lots: half of the fruit was incubated at 20°C and 90% RH for 12 days and the other half was kept for up to 12 weeks at 5°C and 90% RH.

Effect on gray mold development. The effect of gaseous treatments in the development of gray mold caused by *B. cinerea* on artificially inoculated pomegranate fruit was assessed by measuring disease incidence (percentage of decayed fruits relative to the total number of treated fruits) and severity (lesion diameter) on control and treated fruit. On fruit incubated at 20°C, evaluations were performed every 3 to 4 days for a period of up to 12 days. On fruit stored at 5°C, evaluations were performed every 12 to 14 days for a period of up to 82 days.

Quality of cold-stored fruit. In parallel with the second experiment described above, noninoculated fruit were treated for 48 h with air (control), 95 kPa CO₂, or the combination 30 kPa O₂ + 70 kPa CO₂ at 20°C and stored at 5°C for 12 weeks. External and internal fruit quality was determined at harvest (initial quality) and at the end of the 12-week cold-storage period, according to the following procedures. Weight loss was measured on 30 fruit per treatment. Individually labeled fruit were weighed at the beginning of the experiment and at the end of the cold-storage period. The results were expressed as the percent loss of initial weight. Skin color was measured on 20 fruit per treatment with a Minolta Chroma Meter (Model CR-300; Minolta USA Co., Ramsey, NJ) according to the Commission Internationale de l'Eclairage. Because Mollar de Elche pomegranate fruit are characterized by their nonuniform reddish-yellow rind color, measurements were taken at three equidistant points in the equator of each fruit and described as the three independent attributes of lightness (*L**), red color direction (*a**), and yellow color direction (*b**). For each gaseous treatment, arils from three replicates of five fruit each were pooled and pressed through cheesecloth to extract the juice, and the following juice properties were determined. Soluble solids content (SSC) was obtained with a digital refractometer (Model DR-101; Optic Ivymen System, Barcelona, Spain) and expressed as percentage (°Brix). Titratable acidity (TA) and pH were determined with an automatic titrator (Model T50; Mettler Toledo, Columbus, OH). TA was determined from a 5-ml aliquot from each replicate juice by titration with 0.1 N NaOH to pH 8.1 and expressed as grams of citric acid per 100 ml. Maturity index (MI) was calculated as the SSC/TA ratio. To calculate percent juice yield, arils from each replicate were weighed, then squeezed, and the unfiltered obtained juice was also weighed (juice weight × 100/arils weight).

The effect of gaseous treatments on pomegranate sensory quality was assessed before and after the cold storage by 8 to 10 trained judges. Panelists rated aril flavor on a 9-point scale, where 1 to 3 represented the range of very poor to poor flavor, usually with presence of off-flavors; 4 to 6 the range of acceptable flavor; and 7 to 9 the range of good to excellent flavor. Presence of off-flavors was

evaluated using a 5-point intensity scale, where 1 indicated the absence of off-flavor and 5 the detection of very intense off-flavor. Each judge evaluated one sample per treatment (random selection of arils from 15 fruit/treatment). Samples were presented to panelists in trays labeled with three-digit random codes and served at room temperature ($25 \pm 1^\circ\text{C}$). Spring water was provided for rinsing between samples.

Statistical analysis. Disease incidence and severity and fruit quality data were subjected to two-way analysis of variance (ANOVA) using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA), with gaseous treatment composition and temperature or time of exposure as factors, depending on the experiment. Prior to ANOVA, disease incidence data had been transformed to the arcsine of the square root of the proportion of decayed fruit. No significant interaction in the two-way ANOVA was found between gaseous treatment (GT) and exposure temperature (T) for the quality parameters weight loss, L^* , a^* , TA, SSC, and flavor. Thus, main effects were considered. For the rest of the dependent variables, and because of significant interactions, one-way analyses were performed with the factor GT for each of the two levels of the factor T (i.e., 20 and 35°C). Where appropriate, means were separated by Fisher's protected least significant difference test ($P = 0.05$).

Results

Effect of gaseous exposure on gray mold development. High CO_2 treatments significantly reduced decay incidence on pomegranate incubated at 20°C following a concentration-dependent trend (Fig. 1A). Treatments with 50 and 95 kPa CO_2 reduced the incidence of gray mold by 82 and 92%, respectively, after 5 days of incubation ($P < 0.0001$). After 10 days, at the end of the incubation period,

Fig. 1. Effect of CO_2 -enriched atmospheres on the **A**, incidence and **B**, severity of gray mold on Mollar de Elche pomegranate fruit artificially inoculated with *Botrytis cinerea*, treated 24 h later with air (Control) or 15, 50, or 95 kPa CO_2 at 20°C for 48 h, and incubated at 20°C for 10 days. Within each incubation time, means with the same letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). For disease incidence, analysis of variance was performed on arcsine-transformed data. Untransformed means are shown.

disease incidence was 42.5, 47.5, and 60% lower in 15, 50, and 95 kPa CO_2 -treated fruit, respectively, than on control fruit ($P < 0.0001$). The severity of the disease was also lower on CO_2 -treated fruit, with a reduction of almost 45% in the diameter of the lesions on fruit treated with 95 kPa CO_2 compared with control fruit after 10 days of incubation at 20°C ($P = 0.0028$; Fig. 1B). In contrast, treatments with high O_2 did not significantly reduce gray mold development. After 11 days of incubation at 20°C , disease incidence and severity were 100% and about 30 mm, respectively, on both control fruit and fruit treated with 30 or 45 kPa O_2 (Fig. 2). The combination of 45 kPa O_2 + 15 kPa CO_2 slightly reduced gray mold incidence ($P = 0.0077$) and severity ($P = 0.0056$) after 4 days of incubation at 20°C , with no significant reduction thereafter (Fig. 2).

Exposure to 95 kPa CO_2 at 20°C for 48 h reduced gray mold incidence by 80 and 50% after 2 and 5 days of incubation at 20°C , respectively, while exposure to 30 kPa O_2 + 70 kPa CO_2 significantly reduced the incidence after 2 days ($P < 0.0001$) but not after 5 days (Fig. 3A). At the end of the incubation period, after 12 days at 20°C , both gaseous treatments reduced lesion diameter; however, this reduction was only significant for fruit treated with 95 kPa CO_2 ($P = 0.0484$; Fig. 3B). Exposure of fruit to air, 95 kPa CO_2 , or 30 kPa O_2 + 70 kPa CO_2 for 48 h at 35°C significantly reduced gray mold incidence after the first 3 days of incubation with respect to control fruit exposed to air at 20°C ($P = 0.0082$), making this gaseous mixture the most effective treatment. However, after 12 days of incubation, incidence was only reduced (by 22%) by the mixture (Fig. 3C). In contrast, all treatments (air, 95 kPa CO_2 , and the combination 30 kPa O_2 + 70 kPa CO_2) applied at

Fig. 2. Effect of CO_2 - and O_2 -enriched atmospheres on the **A**, incidence and **B**, severity of gray mold on Mollar de Elche pomegranate fruit artificially inoculated with *Botrytis cinerea*, treated 24 h later with air (Control), 30 kPa O_2 , 45 kPa O_2 , or 40 kPa O_2 + 15 kPa CO_2 at 20°C for 48 h, and incubated at 20°C for 11 days. Within each incubation time, means with the same letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). For disease incidence, analysis of variance was performed on arcsine-transformed data. Untransformed means are shown.

35°C significantly reduced disease severity during the entire incubation period ($P < 0.05$ for every incubation period), with reductions in lesion diameter of about 30 to 40% after 12 days of incubation at 20°C (Fig. 3D).

The effect of gaseous treatments on inoculated, treated, and cold-stored pomegranate fruit is depicted in Figure 4. Exposure to 95 kPa

CO₂ or 30 kPa O₂ + 70 kPa CO₂ at 20°C for 48 h similarly reduced gray mold incidence during the first 4 weeks of storage at 5°C (reductions of 70 and 40% after 12 and 26 days of storage, respectively). However, after 40 days of cold storage and thereafter, disease incidence was only significantly reduced by the mixture 30 kPa O₂ + 70 kPa CO₂ ($P < 0.05$ for every cold-storage period; Fig. 4A). Gray

Fig. 3. Impact of exposure temperature on the effect of CO₂- and O₂-enriched atmospheres on the **A** and **C**, incidence and **B** and **D**, severity of gray mold on Mollar de Elche pomegranate fruit artificially inoculated with *Botrytis cinerea*, treated 24 h later with air, 95 kPa CO₂, or 30 kPa O₂ + 70 kPa CO₂ for 48 h at 20°C (A and B) or 35°C (C and D), and incubated at 20°C for 12 days. For gaseous treatments applied at 35°C (C and D), an additional control treatment of air at 20°C was included. Within each incubation time, means with the same letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). For disease incidence, analysis of variance was performed on arcsine-transformed data. Untransformed means are shown.

Fig. 4. Impact of treatment temperature on the effect of CO₂- and O₂-enriched atmospheres on the **A** and **C**, incidence and **B** and **D**, severity of gray mold on cold stored Mollar de Elche pomegranate fruit. Fruit was artificially inoculated with *Botrytis cinerea*, treated 24 h later with air, 95 kPa CO₂, or 30 kPa O₂ + 70 kPa CO₂ for 48 h at 20°C (A and B) or 35°C (C and D), and stored at 5°C for 12 weeks. For gaseous treatments applied at 35°C (C and D), an additional control treatment of air at 20°C was included. Within each storage time, means with the same letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). For disease incidence, analysis of variance was performed on arcsine-transformed data. Untransformed means are shown.

mold severity was significantly reduced by both gaseous treatments for the whole storage period at low temperature ($P < 0.003$ for every cold-storage period; Fig. 4B). Similarly, when these treatments were applied at 35°C, only the combination 30 kPa O₂ + 70 kPa CO₂ significantly reduced gray mold incidence for the entire cold-storage period, although the reductions were lower as the storage time increased ($P < 0.05$ for every cold-storage period; Fig. 4C). Gray mold severity was significantly reduced by all three heat treatments (air, 95 kPa CO₂, and 30 kPa O₂ + 70 kPa CO₂) after 40 days at 5°C ($P = 0.01$) but only by 95 kPa CO₂ at the end of the cold-storage period (after 82 days; Fig. 4D). When exposure to one of the most effective gaseous treatment (95 kPa CO₂ at 20°C) was tested for only 24 h instead of 48 h, no significant differences between control and treated pomegranate fruit were observed on fruit either incubated at 20°C for 12 days or stored at 5°C for 12 weeks (data not shown).

Effect of gaseous exposure on fruit quality. Two-way ANOVA with the factors GT and T (Supplementary Table S1) showed no significant interactions for the dependent variables weight loss, L^* , a^* , TA, SSC, and flavor. Hence, main effects were studied for these external and internal quality attributes (Table 1). In contrast, significant interactions between GT and T were found for the quality variables b^* , pH, MI, juice yield, and off-flavor; thus, different one-way ANOVA were performed for GT at 20 and 35°C (Table 2).

There were no differences in weight loss after 12 weeks of storage at 5°C between control and gas-treated fruit. All fruit lost around 13 to 14% fresh weight at the end of the storage period (Table 1). Although the external color of the pomegranate rind was significantly affected by gaseous exposure, the practical impact of such modifications was low. When high CO₂ and O₂ treatments were carried out at 20°C, there were no significant changes from color at harvest. However, when treatments were performed at 35°C, b^* value decreased after 12 weeks of cold storage for all treatments but only from values

of 29 to values of 24 to 26 (Table 2). There were significant differences among treatments in L^* and a^* values regardless of temperature. The 30 kPa O₂ + 70 kPa CO₂ treatment significantly reduced L^* value (from 58 to 55) and increased a^* value (from 18 to 20) compared with air (control) or 95 kPa CO₂ treatments ($P < 0.05$; Table 1). Moreover, when fruit were treated for 48 h at 35°C, the 30 kPa O₂ + 70 kPa CO₂ treatment significantly reduced b^* value (from about 26 to 23) compared with air and 95 kPa CO₂ treatments (Table 2), indicating that, during cold storage, this treatment reduced luminosity and the intensity of yellow color, although very slightly. In general, the quality attributes of pomegranate juice were significantly modified during storage at low temperature. pH of juice from fruit treated with 95 kPa CO₂ at 20°C was 5.5, higher than that of fruit treated with air or 30 kPa O₂ + 70 kPa CO₂ (4.5) (Table 2). Fruit treated with 95 kPa CO₂ had a significantly lower TA than fruit treated with air or 30 kPa O₂ + 70 kPa CO₂, regardless of treatment temperature (Table 1), and a higher MI at both exposure temperatures (Table 2). Gaseous treatments had no effect on SCC and, although this parameter was significantly affected by the temperature at which they were applied ($P < 0.05$), the magnitude of the difference was practically irrelevant (15.77 and 15.13% for fruit treated at 20 and 35°C, respectively; Table 1). Juice yield was lower in fruit treated with 30 kPa O₂ + 70 kPa CO₂ at 20°C (57%), whereas it was not significantly different among treatments performed at 35°C (Table 2). Although overall fruit flavor was not affected by exposure temperature (Table 1), the presence of off-flavor significantly increased when the 95 kPa CO₂ atmosphere was applied at 35°C (Table 2).

Discussion

Results from this research revealed that exposure of Mollar de Elche pomegranate inoculated with *B. cinerea* to CO₂-enriched atmospheres at room temperature (20°C) for 48 h reduced gray mold

Table 1. Main effects of the factors gaseous treatment and exposure temperature on external, internal, and sensory quality attributes of mature Mollar de Elche pomegranate fruit after storage at 5°C for 12 weeks

Factors	Quality attributes ^z					
	WL (%)	Skin color L^*	Skin color a^*	TA (% citric acid)	SSC (%)	Flavor (1-to-9 scale)
Gaseous treatment						
Air	14.13 a	58.19 b	18.08 a	0.16 b	15.31 a	6.58 b
95 kPa CO ₂	13.48 a	58.47 b	17.73 a	0.07 a	15.56 a	5.19 a
30 kPa O ₂ + 70 kPa CO ₂	13.31 a	55.09 a	19.95 b	0.15 b	15.48 a	6.12 b
Exposure temperature (°C)						
20	13.38 a	58.15 b	18.21 a	0.13 a	15.77 b	6.28 a
35	13.86 a	56.35 a	18.96 a	0.13 a	15.13 a	5.65 a

^z Abbreviations: WL = weight loss; L^* and a^* = Commission Internationale de l'Eclairage color parameters; TA = titratable acidity; SSC = soluble solids content; Flavor = qualitative scale used by panelists to rate fruit flavor, where 1 to 3 very poor to poor, 4 to 6 acceptable, and 7 to 9 good to excellent. For each factor and quality attribute, means in columns with the same letter are not statistically different according to Fisher's protected least significant difference test ($P = 0.05$).

Table 2. Individual effects of the factors gaseous treatment and exposure temperature (Temp) on external, internal, and sensory quality attributes of mature Mollar de Elche pomegranate fruit after storage at 5°C for 12 weeks

Temp (°C), gaseous treatment	Quality attributes ^z				
	Skin color b^*	pH	MI	Juice yield (%)	Off-flavor (1-to-5 scale)
20					
At harvest	25.61 ± 3.65 ^b	4.59 ± 0.03	89.25 ± 6.45	...	1 ± 0
Air	25.93 a	4.66 a	89.19 a	67.64 b	1.00 a
95 kPa CO ₂	25.45 a	5.56 b	288.48 b	71.36 b	1.33 a
30 kPa O ₂ + 70 kPa CO ₂	25.38 a	4.65 a	105.48 a	56.88 a	1.44 a
35					
At harvest	28.99 ± 5.12	4.58 ± 0.02	78.43 ± 2.06	...	1.16 ± 0.50
Air	25.92 b	4.65 a	106.40 a	62.27 a	1.25 a
95 kPa CO ₂	26.49 b	4.78 b	193.77 b	57.54 a	2.44 b
30 kPa O ₂ + 70 kPa CO ₂	23.61 a	4.89 c	102.54 a	66.68 a	1.44 a

^z Abbreviations: b^* = Commission Internationale de l'Eclairage color parameter; MI = maturity index = SSC/TA, SSC = soluble solids content, TA = titratable acidity; Off-flavor = qualitative scale used by panelists to rate presence of off-flavor, where 1 = absence and 5 = very intense. Values are means ± standard deviation (for b^* , $n = 20$; pH, MI, and juice yield, $n = 3$; off-flavor, $n = 10$). For each temperature and quality attribute, means in columns with the same letter are not statistically different according to Fisher's protected least significant difference test ($P = 0.05$).

development in a concentration-based manner. Shorter exposure times, however, did not prevent fungal growth. On the other hand, short-term treatments with high O₂ were less effective than high CO₂ treatments, and only when high O₂ was combined with elevated CO₂ was gray mold development significantly inhibited. Among the different gaseous treatments tested, 95 kPa CO₂ and the mixture 30 kPa O₂ + 70 kPa CO₂ were selected as the most promising and used in further experiments to assess the integration with heat treatments and the effect on the quality of cold-stored pomegranate fruit. The effect of these gaseous treatments reducing decay caused by *B. cinerea* on pomegranate fruit is probably due to direct toxicity of O₂ and CO₂ to the fungus. CA and MA storage with high levels of CO₂ have been extensively used to inhibit or delay the development of postharvest pathogens due to both their direct toxicity to fungi and indirect effect on fruit physiology, delaying fruit ripening and senescence (Kader 1986). It has also been reported that exposure to high O₂ atmospheres prevented anaerobic and aerobic microbial growth and inhibited enzymatic browning in vegetables (Day 2001). However, it has been suggested that the direct toxicity of elevated CO₂ is higher than that of elevated O₂ (Amanatidou et al. 1999; Wszelaki and Mitcham 2000). Our results are in agreement with these previous reports.

The main shortcoming of a control strategy based on short-term treatments with CO₂ or O₂ is the low persistence of their positive effects. Our results showed that the effect of elevated CO₂ and O₂ on *B. cinerea* inoculated on pomegranate fruit was primarily fungistatic rather than fungicidal, because disease incidence and severity significantly increased with incubation time. This limited duration of the inhibitory activity of the treatments could be expected for CO₂ treatments, because this gas is generally known to be fungistatic. For instance, De Vries-Paterson et al. (1991) found that exposure of sweet cherry fruit inoculated with *Monilinia fructicola* to high CO₂ levels delayed postharvest decay but did not prevent it. Another study reported that treatments with 40 kPa O₂ + 15 kPa CO₂ or 100 kPa O₂ inhibited mycelial growth of *B. cinerea* in vitro but there was not residual inhibition upon removal of the cultures from the atmosphere (Wszelaki and Mitcham 2000). In addition to their direct effect on fungal growth, heat treatments or exposure to high CO₂ or O₂ atmospheres can lead to induced resistance against postharvest pathogens in some fruit species (Terry and Joyce 2004). Prusky et al. (1991) showed that the application of 30% CO₂ for 24 h delayed the symptoms caused by *C. gloeosporioides* in avocado, and demonstrated that this CO₂ treatment induced an increase in the concentration of an antifungal diene (hydroxy-4-oxo-heneicosa-12,15-diene) that led to such arrest of symptoms. Thus, a better understanding of the physiological changes induced by high CO₂ and O₂ on fresh fruit would certainly be of great importance to improve the effectiveness and prolong the inhibitory effect of short-term gaseous treatments.

Due to their relative effectiveness, low cost, and easy application, postharvest heat treatments are the most common physical method to control postharvest diseases of fresh horticultural produce. They are part of many integrated control strategies because their appropriate combination with other antifungal means (physical, chemical, or biological) has often resulted in additive or synergic effects (Mulas and Schirra 2007; Palou 2009; Schirra et al. 2000). On Mollar de Elche pomegranate, Artés et al. (2000) observed that a curing treatment of 3 days at 33°C significantly reduced decay on fruit stored at 5°C for 3 months. Previous work in our laboratory showed that exposure of artificially inoculated citrus fruit to short-term CO₂- or O₂-enriched atmospheres at 33°C markedly inhibited postharvest green and blue molds caused by *Penicillium digitatum* and *P. italicum*, respectively (Montesinos-Herrero et al. 2012a), and was also beneficial to maintain fruit quality after long-term cold storage (Rojas-Argudo et al. 2010). However, in the present work with pomegranate, the combination of high CO₂ or O₂ with heat (35°C for 48 h) did not improve the effectiveness of gaseous treatments against gray mold and, in general, the effectiveness of treatments at 35°C was similar or lower than that of treatments at 20°C, both on fruit incubated at 20°C and stored at 5°C. The fungistatic effect

of exposure to CO₂- and O₂-enriched atmospheres was confirmed on cold-stored pomegranate fruit, and no negative impact of gaseous treatments applied at 20°C on fruit quality was observed after 12 weeks of storage at 5°C. In general, and in agreement with previous reports (Defilippi et al. 2006), gas composition did not considerably affect external color of pomegranate. Weight loss was generally high but it was not affected by exposure to high CO₂ and O₂. In contrast, a significant effect on some internal quality attributes was observed. Fruit treated with 95 kPa CO₂ at 20 or 35°C had significantly higher pH and MI than control fruit. Lower acidity has been associated to exposure to elevated CO₂ (Kader 1986). This pH increase could be a direct reaction by plant tissue to counteract the acidic effect of CO₂ or an indirect response of fruit metabolism to CO₂. In either case, this lower acidity and increased MI affected fruit flavor independently of treatment temperature, and fruit treated with 95 kPa CO₂ were rated as the worst in the sensory evaluation but still in the range of acceptable commercial quality. When this treatment was applied at 35°C, the level of off-flavors significantly increased. Similar results have been reported for mandarin orange fruit treated for 24 h with 95 kPa CO₂ at 33°C (Rojas-Argudo et al. 2010). These authors observed that this gaseous treatment increased peel pitting, as well as acetaldehyde and ethanol contents, resulting in an induction of off-flavor and a decrease of overall fruit quality. It has been shown that excessive concentrations of CO₂ may harm some commodities, inducing cell damage and oxidative stress, making the fruit more susceptible to postharvest diseases. Nevertheless, the negative effect of high CO₂ levels will depend on the commodity susceptibility to CO₂, duration and temperature of the treatment, and O₂ concentration (Blanch et al. 2012; Kader 1986). In the present study, although no visible external rind damage was observed after 12 weeks of storage at 5°C, the 95 kPa CO₂ treatment applied at 35°C might have been too aggressive and induced physiological changes that accelerated the senescence process, leading to off-flavor accumulation, loss of flavor, and increased susceptibility to decay. It appears from the results that this negative effect could be avoided by performing CO₂ treatments at 20°C or increasing the concentration of O₂, as shown by the lower impact on some fruit quality attributes of the combined atmosphere 30 kPa O₂ + 70 kPa CO₂. Likewise, no substantial benefits in terms of disease reduction or positive effects on pomegranate quality were observed when this mixture was applied at 35 instead of 20°C.

Overall, the results from this research indicated that 48 h of exposure to CO₂- and O₂-enriched atmospheres at room temperature could be a suitable treatment to extend Mollar de Elche pomegranate storage life at nonchilling temperatures by reducing decay caused by *B. cinerea* without negatively affecting fruit quality. The integration of these gaseous treatments with heat was not synergistic, and this is a factor that may facilitate commercial adoption because implementation and application costs of heated atmospheres would be considerably lower. Although, in some tests, especially with pomegranate fruit stored at 5°C, the application of the gaseous mixture 30 kPa O₂ + 70 kPa CO₂ was slightly more effective in reducing gray mold than the application of 95% CO₂, the latter would be a preferred treatment at the commercial scale because of simpler installation and lower operational costs. Experiments with naturally infected fruit should be carried out to estimate accurately the potential of this treatment in real market circumstances. Further research should focus on the physiological basis of the beneficial effect of short-term high CO₂ or O₂ treatments on pomegranate fruit. This knowledge may certainly help to improve this technology and extend the inhibitory effect of the treatments. In spite of the limited duration of the positive effects of gaseous exposure, the significant gray mold reductions observed in this work after periods of 7 to 12 days at room temperature (20°C) could be of great value for the Spanish pomegranate industry because the usual destination markets (domestic and EU export markets) are reached in shorter periods of time. Because no chemical fungicides are currently registered in Spain for postharvest use on pomegranate, the implementation of integrated disease management programs to satisfactorily control decay and extend fruit shelf-life is a priority for the sector. Integrated strategies should consider adequate

spray programs in the orchard to reduce fungal inoculum and quiescent infections in combination with appropriate postharvest antifungal treatments. The gaseous treatments reported here leave no chemical residues on or in fruit and could be combined with the application of other nonpolluting control alternatives such as food preservatives or generally recognized as safe compounds, plant extracts, or antifungal natural edible coatings. On the other hand, the commercial adoption of these gaseous treatments could be feasible despite the high installment and maintenance costs, because large storage facilities are already available in many Spanish pomegranate packing-houses and similar treatments have been successfully implemented for other purposes.

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