



# Prestorage application of high carbon dioxide combined with controlled atmosphere storage as a dual approach to control *Botrytis cinerea* in organic ‘Flame Seedless’ and ‘Crimson Seedless’ table grapes



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## ABSTRACT

Pre-storage application of 40% CO<sub>2</sub> at 0 °C for 24 or 48 h and controlled atmosphere (12% O<sub>2</sub> + 12% CO<sub>2</sub>) storage at 0 °C for up to eight weeks on decay control and quality of organic ‘Flame Seedless’ and ‘Crimson Seedless’ table grapes were studied as a postharvest disease control alternative. To simulate different potential field conditions, these organic treatments were applied to organic-grown grapes that were naturally infected (without inoculation), surface inoculated (berries inoculated by spraying with a conidia suspension), and nesting inoculated (clusters inoculated by placing in the middle an artificially infected berry) with the pathogen *Botrytis cinerea*, the cause of grape gray mold. Under these three conditions, a 40% CO<sub>2</sub> for 48 h pre-storage treatment followed by controlled atmosphere reduced the gray mold incidence from 22% to 0.6% and from 100% to 7.4% after four and seven weeks, respectively. High CO<sub>2</sub> pre-storage alone limited botrytis incidence in both naturally and artificially infected grapes, but was more effective when combined with CA. These treatments did not affect visual or sensory fruit quality. Exposure to high CO<sub>2</sub> for 24 or 48 h effectively inhibited mycelial growth of *B. cinerea* in PDA plates incubated at 22 °C for up to 72 h. Conidia germination in PDA plates was reduced ~60% after 12 h incubation. *In vitro* studies demonstrated a fungistatic effect, but further studies on the mechanism of action could improve treatment performance. This novel high CO<sub>2</sub> initial fumigation followed by controlled atmosphere during storage or transportation could be a commercially feasible alternative for postharvest handling of organic and conventional table grapes. Our results encourage validating this combined physical treatment in other cultivars and under commercial conditions.

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## 1. Introduction

Fungal decay is the primary cause of rapid and extensive postharvest deterioration in table grapes. The major disease is gray mold caused by *Botrytis cinerea*, which can grow at temperatures as low as –0.5 °C and spreads rapidly by means of aerial mycelium among the berries (Crisosto and Mitchell, 2002). If grapes are not treated after harvest and/or during storage, gray mold infection can affect the majority of the berries, leading to substantial losses.

The three main mechanisms of infection that cause postharvest losses to botrytis in table grapes are: latent infection during growth and development before harvest; infection from conidia present in the air and/or on the surface of the berries; and nesting infection

from visibly infected berries that have escaped removal during packaging (Luvisi et al., 1992; Lichter et al., 2006). The contribution from each type of infection varies, depending on the incidence and severity of field infections, storage conditions, and inoculum amounts. However, maturity and length of storage increase a berry’s susceptibility to infection and decay symptoms during postharvest handling. Decay is visible as “slip-skin” (separation of the skin from the flesh upon touch) accompanied by red-brown coloration (easily scored on white varieties), resulting in formation of “nests” (Luvisi et al., 1992; Chervin et al., 2012).

Efficient postharvest control of gray mold is commonly achieved using weekly applications of sulfur dioxide (SO<sub>2</sub>) gas in storage rooms, following an initial SO<sub>2</sub> application prior to cold storage (Luvisi et al., 1992), and/or by packing the grapes with a pad or generator containing sodium metabisulfite, which releases SO<sub>2</sub> when hydrated by water vapor inside the boxes (Droby and Lichter, 2004). A series of SO<sub>2</sub> fumigations are the commercial practice applied in California and the SO<sub>2</sub> generating pad is used in Chile, Israel, South Africa, and by most California shippers who transport

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long distance (Droby and Lichter, 2004). Despite its efficacy at controlling gray mold, the SO<sub>2</sub> technology may compromise fruit taste, cause damage to berries (evident as hairline cracks and bleaching), contribute to air pollution, and potentially be corrosive to metal equipment within storage facilities. Sulfite residues cause reactions in people allergic to sulfite. Thus, a tolerance limit of 10 μL L<sup>-1</sup> for sulfite residues in table grapes was established by the U.S. Environmental Protection Agency (Anonymous, 1989). Human health and worker exposure concerns imposed dramatic changes to the amount of SO<sub>2</sub> applied by improving exposure efficiency without creating greater loss (Luvisi et al., 1992; Crisosto and Mitchell, 2002). Neither synthetic fungicides nor SO<sub>2</sub> can be classified as organic, and therefore, these are not allowed on organic grapes (Romanazzi et al., 2012). There are also strict regulations regarding synthetic fungicides and SO<sub>2</sub> residues in important European markets (Nigro et al., 2006). Some alternatives to SO<sub>2</sub> have been studied: application of ethanol (Karabulut et al., 2003), ethanol combined with chitosan or calcium chloride (Romanazzi et al., 2007; Chervin et al., 2009), organic salts (Nigro et al., 2006), controlled atmosphere (Crisosto et al., 2002a,b; Retamales et al., 2003; Artés-Hernández et al., 2004; Chen et al., 2011), or ozone (Palou et al., 2002; Smilanick et al., 2010). These methods lack enough support to replace SO<sub>2</sub> as a commercial practice.

The organic vegetables and fruits market has expanded due to the increasing number of consumers demanding healthy food that is free of pesticide residues. In 2009, U.S. sales of organic fruits and vegetables were \$ 7.8 billion (USDA, 2012). The organic system focuses on sustainable production and the use of readily soluble mineral fertilizers and synthetic chemical pesticides is forbidden (Lind et al., 2003). Options for organic technologies controlling gray mold have been evaluated: dipping berries in an ethanol solution (Lichter et al., 2002), heated water or ethanol solutions (Mlikota Gabler et al., 2005), or organic salts (Nigro et al., 2006). The use of a high carbon dioxide (CO<sub>2</sub>) atmosphere to control *B. cinerea* in table grapes has been studied (Crisosto et al., 2002a,b; Sanchez-Ballesta et al., 2006; Chen et al., 2011). Although high CO<sub>2</sub> effectively delays decay development, it also causes rachis browning and off flavors, particularly in early-harvested fruits with low soluble solids concentrations (Crisosto et al., 2002a,b; Retamales et al., 2003). Therefore, organic growers only have two promising commercial treatments currently available to them: pre-harvest application of CaCl<sub>2</sub>, and postharvest ozone atmosphere during storage and transportation (Romanazzi et al., 2012).

In preliminary studies (Teles, 2013), we applied various CO<sub>2</sub> concentrations for 24 or 48 h and examined the effect on nesting formation on inoculated grapes. These results led to the selection of 40% CO<sub>2</sub> applied for 24 or 48 h to replace the SO<sub>2</sub> initial fumigation. Thus, our objective was to test our combined organic approach that consisted of an initial high CO<sub>2</sub> application prior to cold storage, followed by controlled atmosphere (12% O<sub>2</sub> + 12% CO<sub>2</sub>) during storage and/or transportation, for their effects on decay incidence and quality of 'Flame Seedless' and 'Crimson Seedless' table grapes. This combined approach can be a reliable alternative to SO<sub>2</sub> applications for organic table grape growers and shippers.

## 2. Materials and methods

### 2.1. Plant material

'Crimson Seedless' and 'Flame Seedless' certified organic table grapes (*Vitis vinifera*) were commercially harvested in Delano, CA, immediately transported to the Postharvest Laboratory at UC Davis, and air-cooled to a berry temperature of 1 °C.

### 2.2. Inoculation

Prior to the treatments, 'Flame Seedless' grape clusters were labeled and randomly divided into three groups: (1) naturally infected (without inoculation), (2) surface inoculated (berries inoculated by spraying with a conidia suspension) and (3) nesting inoculation (inoculated with an infected berry). After inoculation, grape clusters (800–1000 g) were placed inside perforated plastic cluster bags and packed into expanded polystyrene (EPS) boxes (nine cluster bags with three bags from each inoculation group). 'Crimson Seedless' clusters were not inoculated because they were harvested in a vineyard with a historic and current high incidence of gray mold.

*B. cinerea* (isolate IC 08, from T.J. Michailides, UC Davis) was incubated on PDA in 85 mm Petri dishes, the conidia were extracted, and the suspension was adjusted to a concentration of  $2 \times 10^4$  conidia/mL and sprayed on the surface of the clusters (Palou et al., 2002). Grapes were air-dried for an hour before the treatment applications. For nesting inoculated fruits, a 10 μL suspension of  $2 \times 10^6$  *B. cinerea* conidia/mL was injected 10 mm deep into the flesh of individual berries using a Hamilton syringe (needle 1 mm external diameter) and incubated at 20 °C for 5 d. One inoculated berry was placed in the middle of the bagged cluster prior to treatment applications.

### 2.3. Treatment

After inoculation, fruits were weighed, packed, and grape boxes were stored at 0 ± 0.5 °C with 95–98% relative humidity (RH) inside 338 L sealed metal tanks with a continuous flow of either air (atmospheric air) for 48 h, or 40% CO<sub>2</sub> (40% CO<sub>2</sub> + 60% N<sub>2</sub>) applied for 24 h or 48 h (initial fumigation). After these pre-cooled storage treatments, grapes were again cold stored in air or controlled atmosphere (CA), which consisted of 12% O<sub>2</sub> + 12% CO<sub>2</sub> + 76% N<sub>2</sub> (Crisosto et al., 2002a). Flow rates and gas mixtures were established using a mixing board with micro-metering valves. Supply and exhaust gas composition was monitored using a CO<sub>2</sub>/O<sub>2</sub> gas analyzer (model 900141, Bridge Analyzers Inc., Alameda, CA). 'Flame Seedless' grapes were stored at 0 ± 0.5 °C with 95–98% RH for four or seven weeks, then removed from the tanks and stored for 48 h at 20 ± 1.0 °C to simulate shelf life (SL). 'Crimson Seedless' grapes were stored for eight weeks under the same conditions.

### 2.4. Decay incidence

Decay incidence in 'Flame Seedless' grapes was measured in naturally infected, surface inoculated, and nesting inoculated grapes after four weeks storage at 0 °C, four weeks at 0 °C + SL, seven weeks at 0 °C, and seven weeks at 0 °C + SL. In naturally infected 'Crimson Seedless' grapes, decay incidence was evaluated after three weeks storage at 0 °C, three weeks at 0 °C + SL, six weeks at 0 °C, six weeks at 0 °C + SL, and eight weeks at 0 °C. Decay incidence was measured as the weight of the decayed berries after removal and expressed as percentage total cluster weight.

For the evaluation at eight weeks 0 ± 0.5 °C + 1, 2, or 3 d, the decayed berries were removed then the remaining healthy berries were stored at 20 °C until the next day's evaluation.

### 2.5. Quality evaluation

Evaluation of grape quality included measurements of weight loss, rachis browning, color, berry firmness (maximum force and percent deformation), soluble solids concentration (SSC), and titratable acidity (TA) in naturally infected fruits. Six cluster bags (replicates) per treatment were evaluated before the treatments and after four and seven weeks storage at 1 °C. Weight loss

was determined with a scale (LC 22016, Sartorius, Elk Grove, IL), accurate to 0.01 g, and expressed as the percentage of initial weight. Rachis browning was evaluated using a subjective scoring in which 1 = green and fresh, 2 = green and partially dry, 3 = dry and brownish green, 4 = dry and brown, and 5 = very dry, brown, and brittle (Mlikota Gabler et al., 2005). Fifteen berries from each replicate were randomly detached from the cluster for color, firmness, SSC, and TA measurements. The surface color of the berries was measured with a Chroma meter (CR400 model, Konica Minolta Optics, Japan) using the CIELAB color system. Color index for red grapes (CIRG) (Carreño et al., 1995) was calculated as  $CIRG = (180 - h^\circ)/(C^* + L^*)$ , where  $L^*$  is the lightness corresponding to a black-white scale (0, black; 100, white),  $h^\circ$  is the hue angle on the color wheel, and  $C^*$  is the Chroma (measuring the intensity of color), beginning from zero (achromatic) as it increases in intensity. Berry firmness was measured using a texture analyzer (TA XT, Stable Micro Systems Ltd., UK) with a 2 mm probe that penetrated to a depth of 6 mm at a speed of  $1 \text{ mm s}^{-1}$ . The maximum force (N) necessary to puncture the skin of an individual berry was measured and berry deformation (%) was determined.

Fifteen berries per replicate were filtered through two layers of cheesecloth to extract the juice. SSC was measured with a refractometer (PR-32 $\alpha$ , Atago, Japan) and the titratable acidity (TA) was determined from the same juice sample by titrating with 0.1 N NaOH to pH 8.2, and expressed as percentage tartaric acid.

## 2.6. Acetaldehyde and ethanol

Acetaldehyde and ethanol concentrations were determined after 'Flame Seedless' grapes were treated with Air, 40% CO<sub>2</sub> for 24 h + air, and 40% CO<sub>2</sub> for 48 h + air, following one or two weeks storage at 0 °C. Samples of all treatments (air and CA stored) were taken at four and seven weeks storage at 0 °C. 5 mL juice was incubated for 1 h at 65 °C in a septum-capped tube (Ke et al., 1991). The acetaldehyde and ethanol concentrations in 1 mL headspace gas were determined using a gas chromatograph (model GC-9 AM; Shimadzu, Kyoto, Japan) with a flame ionization detector (250 °C) and a 5% carbowax on 60/80 Carbowax column (Supelco, Bellefonte, PA). Volatiles were quantified through comparison to known standards and the juice sample concentration was expressed as  $\mu\text{L L}^{-1}$  (Ke et al., 1991). Six replicates per treatment were analyzed.

## 2.7. Sensory triangle test

To test whether high CO<sub>2</sub> followed by CA affected the flavor of 'Flame Seedless' table grapes, grapes cold stored for seven weeks in air or treated with 40% CO<sub>2</sub> for 48 h followed by CA were compared. Twenty untrained panelists participated in a triangle test with three randomized samples of three berries each, one different sample and two alike. The panelists were instructed to eat the berries and identify the odd sample (Meilgaard et al., 1999).

## 2.8. Effect of high CO<sub>2</sub> on *in vitro* mycelial development of *B. cinerea*

The effect of high CO<sub>2</sub> on mycelial growth of *B. cinerea* was assayed in PDA. An 8 mm diameter plug of agar containing mycelia was obtained from the growing edge of 3 d old *B. cinerea* cultures and placed in the center of an 85 mm diameter Petri dish of PDA medium. The Petri dishes were exposed to a  $3.33 \text{ mL s}^{-1}$  flow of 40% CO<sub>2</sub> for 24 or 48 h and the controls were exposed to air at the same flow for 24 or 48 h. The treatments were applied inside 7.8 L tanks stored in a cold room at 0 °C with a RH of 95–98%. Radial growth of *B. cinerea* was recorded at 0, 24, 48, and 72 h after incubation at 22 °C in the dark. To determine whether the effects observed during *in vitro* studies were influenced by the direct impact of CO<sub>2</sub> on

the culture medium, dishes with PDA were exposed to 40% CO<sub>2</sub> as above, prior to inoculation. Relative growth (RG) was estimated for each interval based on the ratio of the mean of the diameter, less 8 mm at each interval, divided by the mean diameter, less 8 mm, of colonies that were not exposed to CO<sub>2</sub> for the same time interval. Relative inhibition growth (RIG) was estimated using the equation  $RIG\% = 100 - RG$ . For each treatment, six replicate dishes were prepared and the experiment was repeated once.

## 2.9. High CO<sub>2</sub> effects on conidial viability *in vitro*

Conidia from 10 to 11 d old colonies of *B. cinerea* were collected by adding 10 mL sterile deionized water to each Petri dish. The density of the conidia suspension was measured with a hemacytometer (Brightline, NY, USA) and adjusted to  $2 \times 10^5$  conidia/mL, then 100  $\mu\text{L}$  suspension was placed onto 85 mm Petri dishes containing 20 mL PDA. The Petri dishes were exposed to a  $3.33 \text{ mL s}^{-1}$  flow of 40% CO<sub>2</sub> for 24 or 48 h, and controls were exposed to air at the same flow for 24 or 48 h. The treatments were applied inside 7.8 L tanks stored in a cold room at  $0 \pm 0.5$  °C and 95% RH. After treatments, the dishes were transferred to an incubation chamber at 22 °C in the dark for 24 h. The extent of germination of 100 conidia per dish was assessed by microscopic observation (200 $\times$ ). Conidia with germ tubes longer than the conidial diameter were considered germinated. For each treatment, six replicate dishes were prepared and the experiment was repeated once.

## 2.10. Statistical analysis

A factorial design was used, with three pre-storage and two storage conditions as factors for six treatments ( $3 \times 2$ ), and six replicates at each evaluation date of 'Flame Seedless'. Each of the six replicates per treatment and evaluation date consisted of one grape cluster of 800–1000 g. Decay incidence data were transformed (arcsin of the square root of the proportion of affected fruit) before the analysis. For mycelial growth and conidial germination, six replicate dishes were prepared for each treatment, the experiment was repeated once, and the results presented are the average of both experiments.

The data were subjected to an analysis of variance (ANOVA) and the means were separated using Tukey's test ( $p \leq 0.01$  or  $p \leq 0.05$ ) or an unpaired *t* test ( $p \leq 0.05$ ).

## 3. Results

### 3.1. Decay incidence

Some two-way interactions between the pre-storage and storage factors were significant; these are indicated in the tables. When the interactions were not significant, the factors effects are discussed in the text. There were no significant differences between untreated (control) and 40% CO<sub>2</sub> for 24 h + CA on naturally infected 'Crimson Seedless' until six weeks cold storage. Decay incidence became important after six weeks, when control grapes had 4.1% decay and treated grapes, only 0.4% decay. After six weeks + SL, treated grapes had ~3% decay while the control reached ~27% (Table 1). The 40% CO<sub>2</sub> for 24 h + CA still reduced decay incidence after 3 d SL at  $20 \pm 1$  °C, simulating commercial marketing after eight weeks cold storage.

During cold storage, the natural incidence of gray mold decay in untreated 'Flame Seedless' grapes increased from 22% to 100% over the seven-week evaluation (Table 2). At four weeks, gray mold was significantly controlled by pre-storage and storage treatments. Grapes stored under CA exhibited approximately seven times less decay (2%) than did grapes stored under air (16%) (data not shown). By 4 weeks + SL, the significantly effect on the reduction of decay became evident with the pre-storage treatment of high CO<sub>2</sub>, as well as the storage conditions. At this time, the decay incidence for berries under air was again significantly higher (72%) than under CA (16%). A similar effect was observed for pre-storage in 40% CO<sub>2</sub> for 48 h alone (40%) compared with the control (56%) (data not shown). By seven weeks, untreated grapes reached 100% decay and interaction between pre-storage and storage treatments was significant ( $p \leq 0.0009$ ). At this time, the best gray mold control treatment was pre-storage with 40% CO<sub>2</sub> combined with CA, followed by air + CA. Grapes from the other treatments were completely decayed by gray mold. After 7 weeks + SL, the decay incidence in all treatments was >90% (Table 2).

**Table 1**

Natural decay incidence (%) in 'Crimson Seedless' table grapes clusters stored at  $0.5 \pm 1^\circ\text{C}$ , 95–98% RH and subjected to an atmosphere of air (control) or pre-stored in 40%  $\text{CO}_2$  for 24 h and then stored in a controlled atmosphere of 12%  $\text{CO}_2 + 12\% \text{O}_2$  (40%  $\text{CO}_2$  for 24 h + CA) for eight weeks cold storage. Finally, to simulate commercial shelf life, the grapes were stored in air for 2 d at  $20 \pm 1^\circ\text{C}$  (SL) or kept at  $20 \pm 1^\circ\text{C}$  from 1 to 3 d, to evaluate the decay process when decayed berries were removed daily.

Treatments	Storage time							
	3 weeks	3 weeks + SL	6 weeks	6 weeks + SL	8 weeks	8 weeks + 1 d	8 weeks + 2 d	8 weeks + 3 d
Control	0.0	1.7	4.1 a	27.2 a	13.3 a	22.0 a	29.1 a	47.2 a
40% $\text{CO}_2$ for 24 h + CA	0.4	0.6	0.4 b	3.3 b	0.2 b	0.4 b	1.8 b	7.5 b
<i>P</i> value	0.3409	0.2697	0.0165	0.0003	0.0001	0.0000	0.000	0.0051

Incidence data were transformed (arcsin of the square root of the proportion of affected fruit) before analysis of variance and Student's *t*-test.

Values followed by the same letters for each assessment time did not differ significantly according to pairwise Student's *t* test ( $p \leq 0.05$ ),  $n = 6$ .

**Table 2**

Natural decay incidence (%) in 'Flame Seedless' table grapes affected by the interaction of high  $\text{CO}_2$  pre-storage (air; 40%  $\text{CO}_2$  for 24 h; 40%  $\text{CO}_2$  for 48 h) and storage (air; CA = 12%  $\text{CO}_2 + 12\% \text{O}_2$ ). The fruits were stored at  $1.0 \pm 0.5^\circ\text{C}$ , 95–98% RH, and then stored in air for 2 d at  $20 \pm 1^\circ\text{C}$  (SL) to simulate commercial shelf life.

Treatment	Storage time			
	4 weeks	4 weeks + SL	7 weeks	7 weeks + SL
Air	22.0	79.0	100.0 a	100.0 a
40% $\text{CO}_2$ for 24 h + air	16.3	59.3	100.0 a	100.0 a
40% $\text{CO}_2$ for 48 h + air	9.0	76.7	100 a	98.7 a
Air + CA	6.3	32.8	46.0 b	98.4 a
40% $\text{CO}_2$ for 24 h + CA	0.2	12.4	23.1 c	94.0 b
40% $\text{CO}_2$ for 48 h + CA	0.6	3.7	7.4 c	90.7 b
Pre-storage <i>P</i> -value	0.0084	0.0451	0.0009	0.0006
Storage <i>P</i> -value	0.0000	0.000	0.0000	0.0000
Pre-storage $\times$ storage <i>P</i> value	0.3636	0.0827	0.0009	0.0338

Statistical analysis was performed with arcsine of the square root transformed data. Values presented are non-transformed means. Values in the column followed by the same letter are not significantly different according to Tukey's test ( $p \leq 0.05$ ),  $n = 6$ .

In surface inoculated grapes, the decay incidence in control grapes increased from 26% at four weeks cold storage to 99% at seven weeks (Table 3). A significant interaction between pre-storage high  $\text{CO}_2$  and storage conditions was observed up to seven weeks cold storage. After 4 weeks, fruits treated with high  $\text{CO}_2 + \text{CA}$  did not exhibit any decay, while the treatments combined with air yielded a decay incidence of 10–34%. At four weeks + SL, the order of decay control, from greatest to least, was 40%  $\text{CO}_2$  for 48 h + CA (4%), 40%  $\text{CO}_2$  for 24 h + CA (7%), and air + CA storage (25%). At the same time, grapes from the remaining treatments had gray mold incidence >93%. After seven weeks cold storage, the 40%  $\text{CO}_2$  for 48 h + CA, 40%  $\text{CO}_2$  for 24 h + CA and air + CA treatments again yielded the highest decay control at 27%, 50%, and 75% infection, respectively, while the other treatments had >95% decay (Table 3). At seven weeks + SL, all treatments exhibited very high botrytis infection rates. Surface inoculated and naturally infected both yielded >95% gray mold incidence.

In nesting inoculated table grapes, the interaction between high  $\text{CO}_2$  pre-storage and storage conditions significantly affected decay incidence (Table 4). By four weeks, grapes treated with 40%  $\text{CO}_2$  for 48 h + CA and 40%  $\text{CO}_2$  for 24 h + CA exhibited 38 times less decay (0.5%) than did grapes stored under air (19%). At four weeks + SL, the decay spread very quickly in grapes stored in air, covering 98% of the clusters. Once more, treatments with 40%  $\text{CO}_2$  for 48 h + CA and 40%  $\text{CO}_2$  for 24 h + CA had the lowest infection rates at 10% and 11%, respectively. After seven weeks cold storage, the order of decay control, from greatest to least, was 40%  $\text{CO}_2$  for 48 h + CA, 40%  $\text{CO}_2$  for 24 h + CA, and air + CA storage, with decay incidence of 11%, 25%, and

32%, respectively. At this time, clusters stored in air exhibited >98% decay incidence (Table 4). After seven weeks + SL, the 40%  $\text{CO}_2$  for 48 h + CA clusters still had less decay, at 84%, than clusters from the other treatments, which were completely covered by decay. Throughout storage, regardless of inoculation method, CA storage significantly reduced decay incidence below that of clusters stored in air (data not shown).

### 3.2. Quality evaluation

At harvest, 'Flame Seedless' grapes had 18.2% SSC, 0.52% tartaric acid, a rachis browning rating of 2.4 (slightly browning), 3.7 N maximum penetration force, and 19.5% deformation, while 'Crimson Seedless' had 20.4% SSC, 0.65% tartaric acid, 4.5 N maximum penetration force, and 17.0% deformation.

The quality of 'Flame Seedless' grape was evaluated during storage. The treatments did not significantly affect rachis browning, SSC, TA, weight loss, or color, these values remained similar to those at harvest (data not shown). The TA after seven weeks cold storage (0.53%) was similar to the TA before storage (0.52%). The grapes retained a CIRG of 4.4 (red) from harvest through storage. After four weeks cold storage, fruits stored in air required 12% less maximum force to penetrate. The 40%  $\text{CO}_2$  for 24 h + CA and 40%  $\text{CO}_2$  for 48 h + CA treatments maintained fruit firmness during four weeks storage. At seven weeks, no significant difference between treatments was detected, but the maximum penetration force was ~20% lower than

**Table 3**

Decay incidence (%) in surface-inoculated 'Flame Seedless' table grapes affected by the interaction of high  $\text{CO}_2$  pre-storage (air; 40%  $\text{CO}_2$  for 24 h; 40%  $\text{CO}_2$  for 48 h) and storage (air; CA = 12%  $\text{CO}_2 + 12\% \text{O}_2$ ). The fruits were stored at  $1.0 \pm 0.5^\circ\text{C}$ , 95–98% RH, and finally, to simulate commercial shelf life, the grapes were stored in air for 2 d at  $20 \pm 1^\circ\text{C}$  (SL).

Treatment	Storage time			
	4 weeks	4 weeks + SL	7 weeks	7 weeks + SL
Air	25.6 a	98.9 a	99.2 a	100.0
40% $\text{CO}_2$ for 24 h + air	33.8 a	93.6 a	95.0 ab	100.0
40% $\text{CO}_2$ for 48 h + air	10.1 b	100.0 a	95.8 a	100.0
Air + CA	0.0 c	24.7 b	75.4 bc	100.0
40% $\text{CO}_2$ for 24 h + CA	0.0 c	6.9 c	50.1 cd	97.4
40% $\text{CO}_2$ for 48 h + CA	0.0 c	4.0 c	27.2 d	95.2
Pre-storage <i>P</i> -value	0.0037	0.0250	0.0002	0.2465
Storage <i>P</i> -value	0.0000	0.0000	0.0000	0.0229
Pre-storage $\times$ storage <i>P</i> value	0.0037	0.0258	0.0197	0.2465

Statistical analysis was performed with arcsine of the square root transformed data. Values presented are non-transformed means. Values in the column followed by the same letter are not significantly different according to Tukey's test ( $p \leq 0.05$ ),  $n = 6$ .

**Table 4**  
Decay incidence (%) in nesting inoculated 'Flame Seedless' table grapes affected by the interaction of high CO<sub>2</sub> pre-storage (air; 40% CO<sub>2</sub> for 24 h; 40% CO<sub>2</sub> for 48 h) and storage (air; CA = 12% CO<sub>2</sub> + 12% O<sub>2</sub>). The fruits were stored at 1.0 ± 0.5 °C, 95–98% RH, and finally, to simulate commercial shelf life, the grapes were stored in air for 2 d at 20 ± 1 °C (SL).

Treatment	Storage time			
	4 weeks	4 weeks + SL	7 weeks	7 weeks + SL
Air	18.3 a	94.4 a	100.0 a	100.0 a
40% CO <sub>2</sub> for 24 h + air	22.7 a	100.0 a	98.3 a	98.8 a
40% CO <sub>2</sub> for 48 h + air	16.7 a	100.0 a	98.3 a	100.0 a
Air + CA	4.1 b	22.2 b	32.3 b	98.3 a
40% CO <sub>2</sub> for 24 h + CA	0.5 c	11.1 bc	25.3 bc	100.0 a
40% CO <sub>2</sub> for 48 h + CA	0.4 c	10.0 c	10.8 c	84.2 b
Pre-storage P-value	0.0188	0.8101	0.0065	0.0032
Storage P-value	0.0000	0.0000	0.0000	0.0075
Pre-storage × Storage P value	0.0035	0.0000	0.0468	0.0000

Statistical analysis was performed with arcsine of the square root transformed data. Values presented are non-transformed means. Values in the column followed by the same letter are not significantly different according to Tukey's test ( $p \leq 0.05$ ),  $n = 6$ .

that required to penetrate the fruits prior to storage (Table 5). After four weeks cold storage, berry deformation was more pronounced in the control (13.6%) than in fruits pre-stored with 40% CO<sub>2</sub> for 24 h (16.7%) or 40% CO<sub>2</sub> for 48 h (15.8%). Storage also affected berry deformation, as grapes stored in air had less deformation (14.7%) than fruits stored in CA (16.0%). After seven weeks storage, the treatments stored in air could not be analyzed due to the high decay incidence. Grapes stored in CA had berry deformation values similar to that at harvest: 19.2% and 19.5%, respectively. A similar situation occurred in 'Crimson Seedless', where quality was not affected by the combined treatment (data not shown).

### 3.3. Acetaldehyde (AA) and ethanol

The AA concentration in fruits stored in air increased from 2.1  $\mu\text{L}\cdot\text{L}^{-1}$  before storage to 4.0  $\mu\text{L}\cdot\text{L}^{-1}$  by week four (Table 6). After pre-storage treatment, grapes treated with 40% CO<sub>2</sub> yielded about four times more AA than did grapes pre-stored under air: 8.8–9.5 and 2.1  $\mu\text{L}\cdot\text{L}^{-1}$ , respectively. After one week cold storage, 40% CO<sub>2</sub> for 48 h + air had the most AA at 18.9  $\mu\text{L}\cdot\text{L}^{-1}$  and 40% CO<sub>2</sub> for 24 h + air had 8.6  $\mu\text{L}\cdot\text{L}^{-1}$ . To measure the effect of 40% CO<sub>2</sub> pre-storage alone, concentrations of acetaldehyde and ethanol were analyzed weekly for four weeks in fruits stored in air. The AA concentration in these grapes dropped with pre-storage at 40% CO<sub>2</sub>, with no significant difference at four weeks among treatments of fruits stored in Air (Table 6). For fruits stored in CA for four weeks, those treated with 40% CO<sub>2</sub> for 48 h + CA had 12.4  $\mu\text{L}\cdot\text{L}^{-1}$  AA, but the concentration fell to 3.0  $\mu\text{L}\cdot\text{L}^{-1}$  after seven weeks cold storage, with no significant difference with 40% CO<sub>2</sub> for 24 h + CA and air + CA. Ethanol was not detected in 'Flame Seedless' organic table grapes before treatment. During four weeks cold storage, the ethanol concentration in Air stored fruits increased from 0 to 100  $\mu\text{L}\cdot\text{L}^{-1}$  (Table 6). Pre-storage with 40% CO<sub>2</sub> for 24 h increased the ethanol concentration from 0 to 79.7  $\mu\text{L}\cdot\text{L}^{-1}$  and for 48 h, from 0 to 228  $\mu\text{L}\cdot\text{L}^{-1}$ . After four weeks storage, 40% CO<sub>2</sub> + air fruit did not have a significantly different ethanol concentration. At this time, 40% CO<sub>2</sub> for 48 h + CA yielded the highest ethanol concentration (486  $\mu\text{L}\cdot\text{L}^{-1}$ ). By seven weeks, fruits stored in air could not be analyzed because of extensive decay and the treatments of fruits stored in CA had no significant differences in ethanol concentrations ( $p \leq 0.1124$ ).

**Table 5**  
Maximum force (N) and deformation of berry (%) in 'Flame Seedless' table grapes affected by the interaction of high CO<sub>2</sub> pre-storage (air; 40% CO<sub>2</sub> for 24 h; 40% CO<sub>2</sub> for 48 h) and storage (air, CA = 12% CO<sub>2</sub> + 12% O<sub>2</sub>). The fruits were stored at 1.0 ± 0.5 °C, 95–98% RH.

Initial quality	Maximum force (N)		Deformation (%)	
	3.73	19.5	4 weeks	7 weeks
Treatment	4 weeks	7 weeks	4 weeks	7 weeks
Air	3.28 b	NE	12.9	NE
40% CO <sub>2</sub> for 24 h + air	3.43 b	NE	15.8	NE
40% CO <sub>2</sub> for 48 h + air	3.51 b	NE	15.3	NE
Air + CA	3.30 b	2.95	14.2	18.4
40% CO <sub>2</sub> for 24 h + CA	3.99 a	3.09	17.6	18.9
40% CO <sub>2</sub> for 48 h + CA	3.89 a	2.83	16.4	20.4
Pre-storage P-value	0.0000	–	0.0000	–
Storage P value	0.0000	–	0.0008	–
Pre-storage × Storage P value	0.0000	0.1582	0.7521	0.4063

NE = not evaluated because of high decay incidence. Values in the column that are followed by the same letter are not significantly different according to Tukey's test ( $p \leq 0.05$ ),  $n = 6$ .

### 3.4. Sensory triangle test

There was no significant difference between the taste of samples stored in Air and CA ( $p \leq 0.01$ ). Untrained panelists could not perceive flavor differences between air and treated 'Flame Seedless' table grapes (40% CO<sub>2</sub> for 48 h + CA) stored seven weeks.

### 3.5. Effect of high CO<sub>2</sub> on in vitro mycelium development and conidial viability of *B. cinerea*

40% CO<sub>2</sub> for 24 or 48 h significantly inhibited *B. cinerea* mycelial growth up to 72 h after treatment ( $p \leq 0.01$ ). The relative inhibition of growth (RIG) was enhanced by increased time at 40% CO<sub>2</sub> ( $p < 0.01$ ). At 24 h, *B. cinerea* treated with 40% CO<sub>2</sub> for 48 h had a RIG of 92% and for 24 h, 69% (Fig. 1). After 72 h incubation, *B. cinerea* treated with 40% CO<sub>2</sub> for 48 h had a RIG of 39%, with 18% for fungi treated for 24 h. Pre-treating the culture dishes with 40% CO<sub>2</sub> before adding mycelial plugs did not affect *B. cinerea* growth (data not shown). After 12 h dark incubation at 22 °C, *B. cinerea* conidia germination was reduced significantly by 40% CO<sub>2</sub> ( $p \leq 0.05$ ), from 93% in the control to 39% or 37% in conidia treated with 40% CO<sub>2</sub> for 24 or 48 h, respectively. After 24 h incubation, no significant differences among treatments were found. Germination in treated conidia was 95% or 96% of the control (data not shown).

## 4. Discussion

Under three postharvest infection conditions (naturally infected, surface inoculated, or nesting inoculated grapes), 40% CO<sub>2</sub> pre-storage + CA limited decay incidence in 'Flame seedless' organic table grapes throughout seven weeks cold storage and throughout eight weeks in 'Crimson Seedless'. We assume that surface inoculated and naturally infected fruits represent a wide range of potential field conditions. Placing infected berries into

**Table 6**

Concentration of acetaldehyde and ethanol ( $\mu\text{L L}^{-1}$ ) in 'Flame Seedless' table grapes affected by the interaction of high  $\text{CO}_2$  pre-storage (air; 40%  $\text{CO}_2$  for 24 h; 40%  $\text{CO}_2$  for 48 h) and storage (air, CA = 12%  $\text{CO}_2$  + 12%  $\text{O}_2$ ). The fruits were stored at  $1.0 \pm 0.5^\circ\text{C}$ , 95 to 98% RH.

Initial quality	Acetaldehyde ( $\mu\text{L L}^{-1}$ )					Ethanol ( $\mu\text{L L}^{-1}$ )				
	2.1	0				0				
Treatments	After treatment	1 week	2 weeks	4 weeks	7 weeks	After treatment	1 week	2 weeks	4 weeks	7 weeks
Air	2.1 b	2.1 c	3.5 b	4.0 bc	NE	0.0 c	0.0 c	69.4 b	100.4 b	NE
40% $\text{CO}_2$ for 24 h + Air	8.8 a	8.6 b	4.7 b	4.4 bc	NE	79.7 b	37.8 b	129.5 b	129.0 b	NE
40% $\text{CO}_2$ for 48 h + Air	9.5 a	18.9 a	16.0 a	5.9 b	NE	228.4 a	187.6 a	255.0 a	75.8 b	NE
Air + CA	NA	NA	NA	2.8 c	2.4	NA	NA	NA	110.2 b	179.3
40% $\text{CO}_2$ for 24 h + CA	NA	NA	NA	5.2 bc	2.4	NA	NA	NA	211.5 b	182.9
40% $\text{CO}_2$ for 48 h + CA	NA	NA	NA	12.4 a	3.0	NA	NA	NA	486.3 a	260.0
Pre-storage P-value	–	–	–	0.0000	–	–	–	–	0.0000	–
Storage P value	–	–	–	0.0002	–	–	–	–	0.0000	–
Pre-storage x Storage P value	0.0000	0.0000	0.0000	0.0001	0.1988	0.0000	0.0000	0.0003	0.0000	0.1124

NA = not analyzed. NE = not evaluated because of high decay incidence. Values in the column followed by the same letter are not significantly different according to Tukey's test ( $p \leq 0.05$ ),  $n = 6$ .

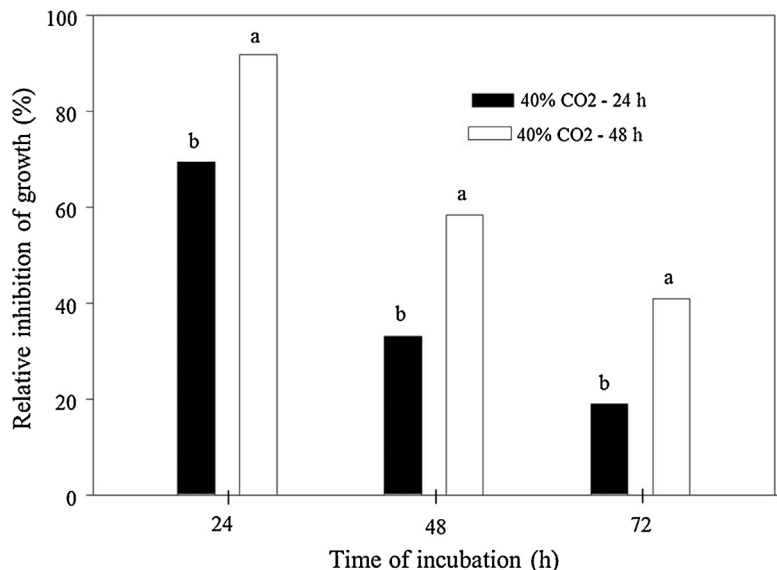
the middle of the cluster simulated a situation where fast packing and/or lack of sanitation occurs. In addition, decay suppression from the combined treatment carried over to the simulated shelf life. According to current U.S. marketing regulations, 0.5% is the maximum decay rate accepted at the shipping point and 1.0% at the receiving point for U.S. No. 1 grade California grapes (Anonymous, 1999). Across three inoculation conditions, 40%  $\text{CO}_2$  for 24 h + CA and 40%  $\text{CO}_2$  for 48 h + CA yielded decay incidence below this maximum: 0.5% in 'Flame Seedless' and 'Crimson Seedless' after four and eight weeks cold storage, respectively, reaching the minimal quality standards for commercial table grapes. Furthermore, at 4 weeks + SL, Flame Seedless' grapes treated with 40%  $\text{CO}_2$  for 48 h + CA still had the lowest decay incidence.

The mode of action of the combined treatment on decay control in table grapes is not fully understood.  $\text{CO}_2$  at high concentrations reduced decay through direct action against *B. cinerea*, including partial inhibition of conidia germination and suppression of mycelial nesting development on grapes with natural and inoculated infections. In our study, the mode of action by which 40%  $\text{CO}_2$  pre-storage + CA induces natural resistance to disease was not evaluated. In grapes and kiwifruit, the efficacy of high  $\text{CO}_2$  or ozone ( $\text{O}_3$ ) treatments in controlling decay resulted from formation of reactive oxygen species associated with stilbene synthase gene

expression and resveratrol accumulation (Sanchez-Ballesta et al., 2006; Romero et al., 2008; Minas et al., 2010), or increased internal ethanol and acetaldehyde to fungal-toxic concentrations (Pesis et al., 1989). Ethanol and acetaldehyde have fungicidal properties and act by damaging membranes and reducing fungal growth (Pesis, 2005). Application of ethanol has allowed good control of gray mold incidence in table grapes (Lichter et al., 2002; Mlikota Gabler et al., 2005; Candir et al., 2012). Latent infections caused by conidia are very difficult to control, even when non-systemic fungicides are applied. Thus, decay will eventually develop in cold storage (Smilanick et al., 1990). Weekly  $\text{SO}_2$  fumigation can kill conidia on the berry surface and prevent spread through nesting on the fruit's surface, but it cannot control latent infection underneath the skin (Luvisi et al., 1992). Therefore, grapes from lots with high latent infection rates are difficult to store successfully for long periods.

Although high  $\text{CO}_2$  pre-treatments prior to cold storage reduced decay early in the cold storage period, decay continued to occur if the fruits were stored or transported in Air. This result emphasizes the synergistic benefit of applying both treatments.

Application of 40%  $\text{CO}_2$  exhibited a direct control over fungal infection. By 24 h, pre-treatment with 40%  $\text{CO}_2$  for 24 or 48 h reduced *in vitro* mycelial growth by 70% or 92%, respectively. When



**Fig. 1.** Relative inhibition growth (%) of *in vitro* mycelial growth of *Botrytis cinerea* treated with 40%  $\text{CO}_2$  ( $0^\circ\text{C}$ , RH 95%) for 24 or 48 h and incubated at  $22^\circ\text{C}$  for up to 72 h. Different letters indicate a significant difference between treatments by Tukey's test ( $p \leq 0.01$ ),  $n = 12$ .

40% CO<sub>2</sub> was applied and fungal development occurred in the air, the fungistatic effect of 40% CO<sub>2</sub> was observed in treated mycelia and conidia, indicating a carry-over effect. A carry-over effect of pre-storage high CO<sub>2</sub> on disease incidence under controlled and commercial conditions has not previously been reported to our knowledge. Dipping grapes in 33% or higher ethanol solutions before storage controlled conidia germination of *B. cinerea* *in vitro* and gray mold in bunches, but did not control mycelial infection. This implies that once germination is established, ethanol is not effective (Lichter et al., 2002). In agreement with our findings, 20% CO<sub>2</sub> reduced *B. cinerea* mycelial growth by 50% and growth decreased linearly at increased CO<sub>2</sub> concentrations (Lichter et al., 2002). High CO<sub>2</sub> application associated with low O<sub>2</sub> concentration yielded stronger control of *B. cinerea* and *Rhizopus stolonifer* spore germination than high CO<sub>2</sub> applied with 21% O<sub>2</sub> (Wells and Uota, 1970). In our *in vitro* test, after 12 h incubation conidia germination was reduced from 93% in the control to 37% in cultures treated with 40% CO<sub>2</sub> for 48 h. However, germination in both control and treated cultures was ~95% at 24 h.

Pre-storage with 40% CO<sub>2</sub> followed by CA reduced loss of firmness, expressed as maximum force to penetrate the fruit, maintaining berry deformation throughout seven weeks cold storage. This treatment did not increase rachis browning or affect color, SSC, or TA, in agreement with previous semi-commercial CA work in which 10–15% CO<sub>2</sub> balanced with air or nitrogen reduced *B. cinerea* decay during long storage periods (Crisosto et al., 2002b; Retamales et al., 2003; Droby and Lichter, 2004), while CO<sub>2</sub> concentrations ≥15% reduced immature grape flavor and appearance while accelerating rachis browning. Thus, this technology should be limited to mature grapes (Crisosto et al., 2002a,b). A short application of 20% CO<sub>2</sub> + 20% O<sub>2</sub> for 72 h in 'Cardinal' table grapes maintained the visual appearance of the rachis, reducing browning and water loss, after 33 d storage at 0 °C (Sanchez-Ballesta et al., 2006). An atmosphere of 4% O<sub>2</sub> + 30% CO<sub>2</sub> reduced the activities of cellulase, polygalacturonase, and peroxidase, reducing berry drop of 'Kyoho' grapes (Deng et al., 2007). CA using low O<sub>2</sub> and high CO<sub>2</sub> has been tested for control of quarantine insects on exported Californian table grapes as a potential alternative to methyl bromide fumigation (Ahumada et al., 1996; Liu, 2013) and CA is actually applied in California as a quarantine treatment in table grapes exported to Australia and New Zealand (Crisosto, personal communication), showing that the use of a short high CO<sub>2</sub> application and CA in table grapes could be economic feasible.

Postharvest application of high CO<sub>2</sub> and/or low O<sub>2</sub> can induce accumulation of fermentative volatiles like ethanol and AA and produce off flavors in fruits. In our study, after seven weeks cold storage, untrained panelists could not identify flavor differences between fruits stored in air and fruits pre-treated with 40% CO<sub>2</sub> for 48 h and stored in CA. Similarly, five trained panelists detected no off flavors in 'Kyoho' grapes stored in high barrier film for two weeks, at which time the ethanol concentration in the fruits had reached ~550 μL L<sup>-1</sup> (Chen et al., 2011). Ethanol vapor applied in a package with low permeability increased the ethanol concentration of 'Red Globe' table grapes from ~280 μL L<sup>-1</sup> at harvest to ~700 μL L<sup>-1</sup> after one month storage at 0 °C, while reducing decay from 51.5% to 4.5%; a trained panel classified the appearance and taste as "like very much" on a hedonic scale (Candir et al., 2012). No off flavors were reported for 'Thompson Seedless' treated with 0.5% O<sub>2</sub> + 45% CO<sub>2</sub> (144 h at 5 °C) and then transferred to air (72 h at 0 °C). In these grapes, ~10 μL L<sup>-1</sup> of AA and ~900 μL L<sup>-1</sup> of ethanol were measured, but consumers were unable to detect flavor differences (Ahumada et al., 1996).

The 40% CO<sub>2</sub> pre-storage for 24 or 48 h, followed by CA, applied to naturally infected 'Flame Seedless' organic table grapes reduced the incidence of decay 55-fold, maintaining USDA commercial standards until four weeks cold storage. Similar, 49-fold reduction

was found in naturally infected 'Crimson Seedless', which maintained commercial standards during eight weeks cold storage. This organic alternative combination treatment provided similar control in conidia- and mycelium-inoculated grapes. High CO<sub>2</sub> pre-storage alone limited incidence of gray mold decay in naturally infected and artificially inoculated 'Flame Seedless' early during storage, but was less effective than the combined treatments. These treatments did not affect visual or sensory quality. The results of *in vitro* experiments and analysis of acetaldehyde and ethanol concentration in stored grapes suggested that high CO<sub>2</sub> acts both directly on *B. cinerea* and also on the grapes, where it increased the acetaldehyde and ethanol concentrations. Thus, high CO<sub>2</sub> short application followed by CA during storage or transportation could be a commercially feasible alternative for postharvest handling of organic grapes. Our results encourage validating this combined organic treatment in other cultivars and studying the mechanism of action to improve performance.

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