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Toxicity of ozone gas to conidia of *Penicillium digitatum*, *Penicillium italicum*, and *Botrytis cinerea* and control of gray mold on table grapes

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ABSTRACT

Penicillium digitatum, *Penicillium italicum*, and *Botrytis cinerea* attack fresh fruit and cause significant postharvest decay losses. The toxicity of ozone (O_3) gas at different relative humidities to control their conidia was determined. Conidia distributed on cover glasses were exposed to an atmosphere containing 200–350 $\mu L\,L^{-1}$ of O_3 gas at 35%, 75%, and 95% relative humidity (RH) at 25 °C. O_3 gas was produced by UV light generators and passed through three 500 mL solutions of saturated $MgCl_2$ (35% RH), NaCl (75% RH), or K_2SO_4 (95% RH). O_3 and RH inside the chamber were monitored. O_3 exposures were quantified as concentration × time products adjusted to 1 h ($\mu L\,L^{-1} \times$ h). After exposure to O_3 for varying periods, the conidia were removed from the chamber, placed on potato dextrose agar and their germination observed. Conidia died more rapidly at higher humidity than at lower humidity, and *P. digitatum* and *P. italicum* were more resistant to O_3 than *B. cinerea*. At 95% RH, 99% of the conidia of *P. digitatum*, *P. italicum*, and *B. cinerea* were incapable of germination after O_3 exposures of 817, 732, and 702 $\mu L\,L^{-1} \times$ h, respectively. At 75% RH, similar inhibition required exposures of 1781, 1274, and 1262 $\mu L\,L^{-1} \times$ h, respectively. At 35% RH, O_3 toxicity declined markedly, and 99% mortality required 11,410, 10,775, and 7713 $\mu L\,L^{-1} \times$ h, respectively. These values can be used to select O_3 gas exposures needed to control these conidia. Conidia of *B. cinerea* were sprayed on to the surface of table grapes and 2 h later the grapes were exposed to 800–2000 $\mu L\,L^{-1} \times$ h of O_3. O_3 at 800 $\mu L\,L^{-1} \times$ h or more reduced the incidence of infected berries by 85% and 45% on 'Autumn Seedless' and 'Scarlet Royal' grapes, respectively. Fumigation with O_3 can control postharvest pathogenic fungi on commodities that tolerate this gas, or it can be applied to disinfect processing equipment and storage rooms when the produce is not present.

1. Introduction

In 1997, an expert panel reviewed the safety and potential food processing use of ozone (O_3), the tri-atomic form of oxygen, and declared O_3 to be generally recognized as safe (GRAS) for food contact applications in the United States (Graham et al., 1997; US-FDA, 1997). Since that time, the GRAS status was affirmed (US-FDA, 2001) and interest in developing O_3 applications in the food industry has increased. O_3 is a naturally occurring substance in the atmosphere and one of the most potent sanitizers against a wide spectrum of microorganisms (Khadre et al., 2001) and it can be used in air or water for postharvest treatments of fresh fruit and vegetables (Karaca and Velioglu, 2007; Palou et al., 2007). In cold storage rooms it can be continuously or intermittently added to the storage atmosphere. Both approaches have recently received considerable commercial interest, especially because of the lack of residues on the produce and new regulatory issues. For example, O_3 use in citrus storage rooms is now common in California to retard the production of conidia on decaying fruit infected with *Penicillium digitatum* or *Penicillium italicum* (Palou et al., 2001), and it has been shown to greatly reduce the spread of *Botrytis cinerea* on stored table grapes (Palou et al., 2002). These authors observed that, although spread of decay by the growth of aerial mycelia was effectively inhibited by 0.3 $\mu L\,L^{-1}$ O_3, conidia on berries in this atmosphere could germinate and infect the fruit, which indicated higher concentrations of O_3 gas were needed to inactivate conidia. Although some of the benefits of O_3 have been established (Palou et al., 2007; Mlikota Gabler et al., 2010), little has been published regarding the quantification of O_3 toxicity to fungal conidia under controlled conditions. Therefore, the objectives of this study were to determine the toxicity of O_3 gas to conidia of *P. digitatum*, *P. italicum* and *B. cinerea*, and further evaluate an O_3 gas treatment on decay incidence of table grapes inoculated with *B. cinerea*.

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2. Materials and methods

2.1. Preparation of conidia

B. cinerea isolate 2004, *P. digitatum* isolate D201, and *P. italicum* isolate 1440 were obtained from the Commodity Protection and Quality Research Unit San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA. The stock cultures were sub-cultured on potato dextrose agar (PDA) and incubated at 24 °C. Conidia from cultures were dispersed onto one side of a cover glass (22 mm × 22 mm, Corning Inc., Corning NY, USA) using a brush to distribute the conidia individually over the entire surface, while minimizing the incidence of conidial clumps.

Before exposure to O_3 gas, conidia were conditioned at each relative humidity in a conditioning chamber identical in size to the O_3 chamber with saturated salt solutions of K_2SO_4, NaCl, or $MgCl_2$ to maintain 95%, 75% or 35% RH, respectively. The conditioning period was 2 h, 12 h, and 12 h, respectively, at 95%, 75% and 35% RH. Because conidia of *B. cinerea* began to germinate if conditioning at 95% RH was 12 h in length, the conditioning period was reduced to 2 h at this RH. All conidia, including the controls not exposed to O_3, were conditioned.

2.2. O_3 system

O_3 gas was generated with three UV light generators (Model CS–1400, Clearwater Tech. Inc., San Luis Obispo, CA, USA). The generated O_3 gas was contained in a cylindrical glass chamber (capacity 10.4 L; 23 cm diameter, 25 cm height) which had a polymethylmethacrylate lid with teflon seals in order to ensure it was gas tight. On the lid there were eight holes which were plugged with silicone stoppers (No. 9.5, Cole Parmer Instrument Co., Vernon Hills, IL, USA) that served as sampling ports; the bottom of the stoppers were modified by the attachment of metal hangers for suspending the cover glasses with fungal conidia within the chambers. Relative humidity (RH) within the O_3 chamber was controlled by passing O_3 gas with a flow rate of 1200 mL min^{-1} through saturated salt solutions of K_2SO_4, NaCl, or $MgCl_2$ to maintain 95%, 75% or 35% RH, respectively. The temperature during the experiment was 25 °C. O_3 gas was constantly monitored within the exposure chamber with a UV-photometric analyzer (Model 450, Advanced Pollution Instrumentation Inc., San Diego, CA, USA) and the O_3 gas concentration inside the chamber was adjusted to 200, 250, or 350 μL L^{-1} ($\pm$10 μL L^{-1}), respectively, for use at 95%, 75%, or 35% RH. A hygrothermograph (Model BDHT, Extech Instruments Corp., Waltham, MA) was present within the chamber confirmed the temperature and RH had reached equilibrium before the O_3 exposures began. O_3 exposure was expressed as a product of the O_3 concentration times the length of exposure in hours ($C \times T$ product, in units μL L^{-1} × h or ppm h^{-1}), by the method of Bond (1984).

2.3. Treatments

The O_3 concentration within the chambers was recorded at intervals of 30 min and remained relatively constant during each exposure. Periodically, cover glasses were removed from the O_3 chamber and the sampling times were varied depending on the fungus and RH%. The O_3 concentration during exposure and at the time of removal was recorded and used for the $C \times T$ product calculation. After O_3 exposure, conidia were transferred by lightly pressing the cover glass with conidia onto PDA. The cover glass was removed and 25 μL of distilled water was added to the conidia and they were spread across the PDA surface. Within each sampling time, 3 replicates were prepared. Petri dishes with conidia were incubated at 20 °C for 18 h. After incubation, 100 conidia per replicate for each treatment were selected randomly and examined for germination by light microscopy. The conidia were counted as germinated if the germ-tube length was greater than their diameter.

Single berries with the pedicel attached of ‘Autumn Seedless’ and ‘Scarlet Royal’ table grapes were surface-sterilized by brief immersion in a 5% (v/v) solution of laundry bleach (5.25% sodium hypochlorite). After they dried, they were inoculated by applying 12,500 conidia/mL of *B. cinerea*, to run-off with a fine mist applied by a compressed air sprayer and air-dried for 2 h at 20 °C prior to O_3 fumigation. For each variety, three replicates of 40 berries each were used. The berries were arranged on the galvanized rack and placed inside a polycarbonate chamber containing approximately 200 μL L^{-1} of O_3. The chamber volume was 1 m deep, 1.3 m high, and 2.5 m in length. O_3 was generated from UV light and monitored continuously as previously described. The grapes were removed after $C \times T$ products of 800, 1200, or 2000 μL L^{-1} × h of O_3 had been applied. After treatment, a wet paper towel was put in each box of grapes, and they were stored for 7 days at 20 °C. After 7 days, the incidence and severity of *B. cinerea* infections were evaluated. Incidence was the percentage of berries with visible infections. Severity of symptoms was ranked according to scale where 0 = no infection, 1 = very small spot, 2 = one infected spot, 3 = two to four infected spots, 4 = < 50% the berry infected and sporulation was evident, and 5 = >50% the berry infected and sporulation was evident.

2.4. Statistical analysis

Within each experiment where conidia were exposed to O_3 gas, the germination of non-treated conidia was also determined as the control and the entire experiment were repeated three times. To normalize the data among experiments, the germination percentage, which varied between 60% and 75%, was corrected within each experiment by multiplying all values by the product 100 divided by the actual percentage of germination among untreated conidia (control). Values of concentration × time product ($C \times T$) of O_3 in units of μL L^{-1} × h^{-1} where 99% mortality occurred (EC_{99}) and lower and upper 95% confidence limits of each estimate were determined by probit analysis (Finney, 1971) using SPSS (release 16.0 SPSS, Inc., Chicago, IL). A one-way ANOVA was applied to the percentage of gray mold infected berries or index values of the severity of symptoms of decay on inoculated table grapes treated with O_3 followed by Tukey’s HSD ($P \leq 0.05$) to separate means. An arcsine transformation was applied to percentage values before analysis. Actual values are shown.

3. Results

The germination of untreated conidia after conditioning at each RH of all three fungi was 60–75%. Conidia died more rapidly during O_3 exposure at higher humidity than at lower humidity, and *P. digitatum* and *P. italicum* were more resistant to O_3 than *B. cinerea* (Fig. 1). At 95% RH, 99% of the conidia of *P. digitatum*, *P. italicum*, and *B. cinerea* were incapable of germination after O_3 exposures of 817, 732, or 702 μL L^{-1} × h, respectively, while at 75% RH, similar inhibition required exposures of 1781, 1274, or 1262 μL L^{-1} × h, respectively, and at 35% required exposures of 11,410, 10,775, or 7713 μL L^{-1} × h, respectively (Table 1; Fig. 2).

On ‘Autumn Seedless’ grapes inoculated with *B. cinerea*, the number of infected grapes was reduced from 92.5% to 5.0% by 800 μL L^{-1} × h of O_3 (Fig. 3). On ‘Autumn Seedless’ grapes that had not been inoculated or treated with O_3, 19.2% developed gray mold from natural inoculum. On ‘Scarlet Royal’ grapes inoculated with *B. cinerea*, the number of infected grapes was reduced from 95% to 53.3% by 800 μL L^{-1} × h of O_3 (Fig. 3). Among ‘Scarlet Royal’ grapes that had not been inoculated or treated with O_3, 53.2% developed gray mold from natural inoculum. O_3-treated grapes that did

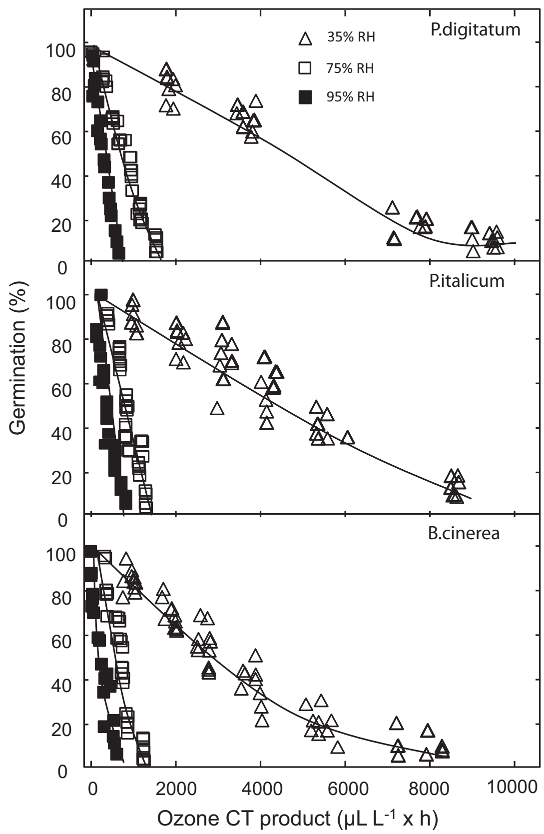

Fig. 1. Germination of conidia of *Penicillium digitatum*, *Penicillium italicum*, and *Botrytis cinerea* after exposure to atmospheres containing 200–350 μL L^{-1} ozone with a relative humidity of 35%, 75%, or 95%. Ozone exposure is shown in concentration $\times$ time products (CT). Each value is the corrected percentage of germination of 100 conidia. Actual germination of untreated conidia was 60–75%.

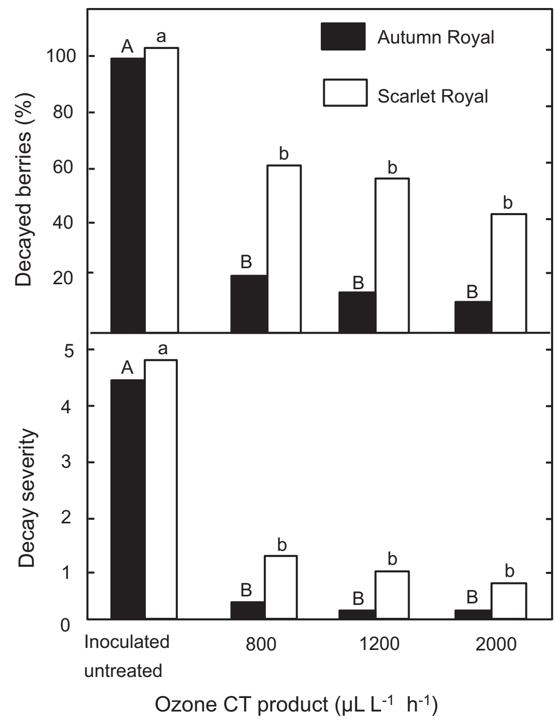

Fig. 3. The incidence of decayed berries and severity of gray mold on 'Autumn Seedless' and 'Scarlet Royal' table grapes after inoculation with conidia of *Botrytis cinerea*, exposure to ozone gas at 800, 1200, or 2000 μL L^{-1} $\times$ h followed by storage at 15 °C for 7 days. Severity values were based on a subjective index, where 0 = no symptoms or signs of gray mold, and 5 = severely decayed berries covered with mycelia. Unlike letters indicate significant differences among values of each cultivar by Tukey's HSD ($P \leq 0.05$).

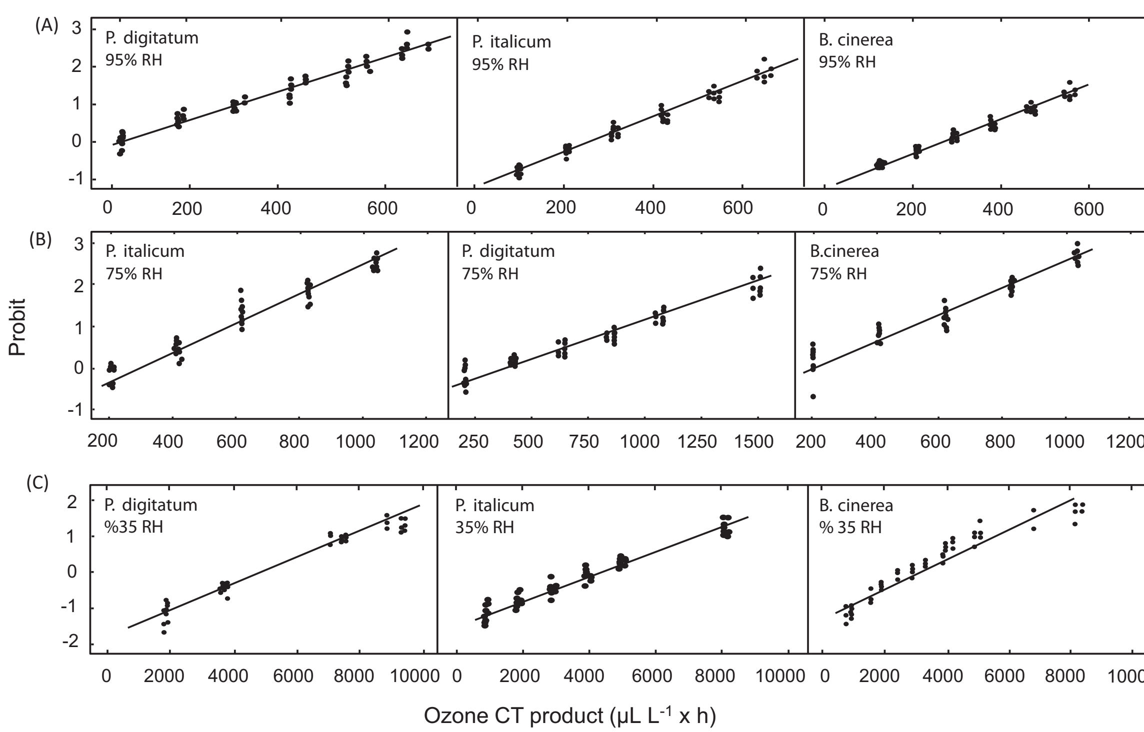

Fig. 2. Probit values of the germination of conidia of *Penicillium digitatum*, *Penicillium italicum*, and *Botrytis cinerea* after exposure to ozone gas, expressed as concentration $\times$ time products (μL L^{-1} $\times$ h), at relative humidities of 95% (A), 75% (B), or 35% (C).

Table 1
Mortality of conidia of three fungi in ozone gas at 35%, 75%, and 95% relative humidity expressed as 1 h $C \times T$ products ($\mu L\,L^{-1}\,h^{-1}$) where 99% mortality occurred (EC_{99}) as estimated by Finney's probit analysis.

Fungus	Relative humidity (%)		
	35	75	95
Penicillium digitatum	11,410 (10,927; 11,962)	1781 (1696; 1879)	817 (720; 899)
Penicllium italicum	10,775 (10,215; 11,437)	1274 (1226; 1328)	732 (702; 766)
Botrytis cinerea	7713 (7149; 8422)	1262 (1201; 1333)	703 (660; 753)

The actual ozone concentration during exposure of the conidia was 200–350 $\mu L\,L^{-1}$. Values in parenthesis are lower and upper 95% confidence limits of each EC_{99} estimate.

develop infections had small, non-sporulating lesions, while the control grapes were covered with aerial mycelium and conidia.

4. Discussion

The effect of O_3 on the growth and virulence of fungal decay pathogens has been reported, but this is one of very few studies where O_3 doses that were required to stop the germination of conidia of these fungi were quantified. Very low concentrations of O_3 (0.3–1.5 $\mu L\,L^{-1}$) caused inhibition of the mycelial growth and sporulation of many fungi, including *B. cinerea* and *Sclerotinia sclerotiorum* on carrots (Liew and Prange, 1994), strawberries (Nadas et al., 2003), and grapes (Palou et al., 2002), *P. digitatum* on citrus fruit (Harding, 1968; Palou et al., 2001), and *Rhizopus stolonifer* on table grapes (Sarig et al., 1996). Krause and Weidensaul (1978) found that exposure to these low O_3 concentrations did not kill but reduced the subsequent virulence of conidia of *B. cinerea*. Reports describing the doses of O_3 to inactivate fungal conidia are few and their conclusions differ. Sharpe et al. (2008) reported conidia of *B. cinerea* were unable to germinate after exposure to only 0.45–0.6 $\mu L\,L^{-1}$ for approximately two days, a concentration $\times$ time product of approximately 22 $\mu L\,L^{-1} \times h$. Korzun et al. (2008) reported significant reductions in the viability of conidia of *Aspergillus niger*, *Cladosporium* spp., and *Stachybotrys* spp. occurred following exposure to O_3 at a concentration of 11.0–12.8 $\mu L\,L^{-1}$ for 4 h, a concentration $\times$ time product of approximately 50 $\mu L\,L^{-1} \times h$. In a preliminary report, Margosan and Smilanick (1998) estimated inhibition of conidia of *B. cinerea*, *Monilinia fructicola*, *P. digitatum*, and *R. stolonifer* required a concentration $\times$ time product of more than 200 $\mu L\,L^{-1}$ under humid conditions and 4000 $\mu L\,L^{-1}$ under dry conditions. For the disinfection of drywall colonized with *Stachybotrys chartarum* or *Aspergillus versicolor*, Schmidt and Rice (2005) applied an O_3 concentration $\times$ time product of approximately 350 $\mu L\,L^{-1} \times h$. Fumigation of stored maize with 50 $\mu L\,L^{-1}$ O_3 for several days, a concentration $\times$ time product of about 5000 $\mu L\,L^{-1} \times h$ reduced colony forming units of *Aspergillus parasiticus* by 63%, while fumigation with 25 $\mu L\,L^{-1}$ O_3 for 5 d did not reduce them significantly (Kells et al., 2001). Reports about other microbes indicate very high O_3 doses can be required to kill those that form spores. Currier et al. (2001) reported that to inactivate the spores of *Bacillus globigi* var. *niger*, 9000 ppm of O_3 for 15 h was required. Reasons values differ so radically can be partially explained by the large impact moisture have on O_3 toxicity and perhaps in the quality of the O_3 generated. For the generation of relatively pure O_3 gas, it is important to use either UV light for its synthesis, or to use very dry air, or preferably oxygen, as a feed gas into a corona discharge generator, because other oxides, particularly of nitrogen, can be generated that contaminate the O_3 produced and alter the toxicity of the discharged gas (Bablon et al., 1991).

A large factor of difference among studies that measured O_3 gas toxicity may also be RH. Our findings corroborate earlier work that increasing humidity is positively correlated with O_3 toxicity. Elford and Ende (1942) reported at a RH below 45%, the disinfectant activity of low concentrations of O_3 gas was negligible. Ewell (1946) later demonstrated O_3 gas killed microbes more rapidly in a humid atmosphere. Kim and Yousef (2000) studied the effects of RH on the inactivation of *Bacillus subtilis* and found the optimum RH was 90–95%. In our study, care was taken to precondition the conidia to the RH used in the O_3 exposure chamber, and the O_3 gas stream itself was humidified before it entered the chamber.

The O_3 exposure values that we found can be used to select O_3 gas exposures needed to control these fungi. Very high concentrations of O_3 were required to inhibit the germination of the conidia, particularly at low RH. Under humid conditions, to control the germination of conidia of *B. cinerea*, the cause of postharvest gray mold, a $C \times T$ product of approximately 700 $\mu L\,L^{-1} \times h$ was needed. A similar $C \times T$ product applied to table grapes inoculated with conidia of this pathogen significantly reduced the subsequent development of gray mold, indicating the values we found approximate those needed in practice to control this pathogen on produce. We speculate that O_3 was less effective on 'Scarlet Royal' than 'Autumn Seedless' grapes because there may have been a higher frequency of latent infections present on them. On 'Autumn Seedless' grapes that had not been inoculated or treated with O_3, 19.2% developed gray mold, while among 'Scarlet Royal' grapes that had not been inoculated or treated with O_3, 53.2% became infected. The higher infection rate on 'Scarlet Royal' may indicate some infections resided within the grape tissue and were not exposed to the O_3 as would conidia residing on the surface of the berries. Latent infections by *B. cinerea* can occur early in the development of the grape and become inactive until the berries are harvested (Keller et al., 2003), although under arid growing conditions characteristic of table grape production areas, many infections are caused by direction penetration by conidia of the mature berries near harvest (Coertze et al., 2001).

Unfortunately, the concentrations of O_3 that inactivated conidia were relatively high and cannot be used without complete containment of the gas and protection of workers from it. Under conditions were O_3 is present during an 8 h workday, O_3 concentrations cannot exceed 0.075 $\mu L\,L^{-1}$ (USEPA, 2008). Equipment to generate and apply very high concentrations of O_3 gas has been developed commercially (Tahoe Food Inc., Sparks NV, USA) and used experimentally to treat harvested table grapes to control postharvest gray mold, caused by *B. cinerea* (Mlikota Gabler et al., 2010), and to reduce *Esherichia coli* O157:H7 on cantaloupe (Selma et al., 2008). In prior work, we found table grape berries tolerated very high rates of ozone without harm, however, the rachis of 'Thompson Seedless' grapes fumigated with ozone 5000 $\mu L\,L^{-1}$ for 1 h was sometimes altered by the development of thin longitudinal darkened lesions (Mlikota Gabler et al., 2010). Rachis injury appeared irregularly, and was not always associated with a particular ozone dose or cultivar. Shimizu et al. (1982) similarly reported the rachis of 'Kyoho' table grapes, and not the berries, were injured at lower ozone rates than those than harmed berries.

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