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Integration of continuous biofumigation with *Muscodor albus* with pre-cooling fumigation with ozone or sulfur dioxide to control postharvest gray mold of table grapes

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ABSTRACT

An integrated approach was evaluated that combined biological and chemical fumigation of table grapes to control postharvest gray mold caused by *Botrytis cinerea*. After fumigation of the grapes with ozone or sulfur dioxide during pre-cooling, the fruit were then exposed to continuous biofumigation by the volatile-producing fungus *Muscodor albus* during storage. Biofumigation was provided by in-package generators containing a live grain culture of the fungus. This grain formulation of *M. albus* survived the initial ozone or sulfur dioxide fumigation, but sulfur dioxide reduced its production of isobutyric acid, an indicator of the production of antifungal volatiles. Gray mold incidence was reduced among inoculated 'Autumn Seedless' grapes from 91.7 to 19.3% by 1 h fumigation with 5000 $\mu L\,L^{-1}$ ozone, and further reduced to 10.0% when ozone fumigation and *M. albus* biofumigation were combined. The natural incidence of gray mold among organically grown 'Thompson Seedless' grapes after 1 month of storage at 0.5 °C was 31.0%. Ozone fumigation and *M. albus* biofumigation reduced the incidence of gray mold to 9.7 and 4.4, respectively, while the combined treatment reduced gray mold incidence to 3.4%. The use of commercial sulfur dioxide pads reduced the incidence to 1.1%. The combination of ozone and *M. albus* controlled decay significantly, but was less effective than the standard sulfur dioxide treatments. Although less effective than sulfur dioxide treatment, ozone and *M. albus* controlled decay significantly, and could be alternatives to sulfur dioxide, particularly for growers complying with organic production requirements.

1. Introduction

Gray mold, caused by *Botrytis cinerea* Pers., the most important postharvest disease of table grapes, is controlled by sulfur dioxide fumigation and storage at −0.5 °C. It is controlled by sulfur dioxide fumigation either at field temperature in fumigation chambers or during initial forced-air cooling of the grapes, followed by 2- to 6-h-long weekly fumigation during cold storage (Harvey and Uota, 1978; Luvisi et al., 1992). In export packages, sulfur dioxide generator sheets are used, which continuously emit a low concentration of gas within the packages during storage when hydrated by water vapor (Droby and Lichter, 2004). Generator pads typically protect the grapes from decay for a period of 2 months, and sometimes longer (Zutahy et al., 2008). While the sulfite residue tolerance is rarely exceeded in commercial practice (Austin et al., 1997), excessive residues can accumulate in wounded or detached berries (Smilanick et al., 1990b). Sulfur dioxide can cause unacceptable bleaching injuries to berries (Crisosto and Mitchell, 2002) and compromise their flavor (Chervin et al., 2005), and can cause food allergies to humans (Tayler et al., 2000). In the USA, it is prohibited from use on certified organic grapes.

Because of issues associated with sulfite residues, sulfur dioxide emissions, and its negative impact on grape quality, safe, effective, and economical alternative strategies to control gray mold are needed (Lichter et al., 2006). Alternatives requiring additional processing are unlikely to be implemented by California table grape growers, who normally pack their fruit into their final commercial packages in the vineyard (Crisosto and Mitchell, 2002).

A novel alternative for controlling fungal diseases is biological fumigation, or biofumigation, with the volatile-producing fungus *Muscodor albus* Worapong, Strobel, and Hess (Strobel et al., 2001;

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Strobel, 2006; Mercier et al., 2007). Isolate 620 of this fungus, which was the first *Muscodor* isolate discovered (Worapong et al., 2001), is currently being developed by AgraQuest Inc., Davis, CA as a biofumigant for agricultural uses (Mercier et al., 2007). The volatiles from *M. albus* isolate 620 are fungicidal to most postharvest pathogens and were used successfully to control storage decay in a number of commodities such as apples (Mercier and Jiménez, 2004), grapes (Mlikota Gabler et al., 2006, 2007), peaches (Mercier and Jiménez, 2004; Schnabel and Mercier, 2006) and lemons (Mercier and Smilanick, 2005).

Continuous biofumigation with *M. albus* effectively controlled gray mold of grapes in many types of packages and storage conditions (Mlikota Gabler et al., 2006, 2007). A developed formulation of *M. albus* consisting of desiccated rye grain colonized by the fungus has to be activated for postharvest use by rehydration (Mercier et al., 2007). This formulation was used in a pad or sachet delivery system for the fumigation of individual shipping boxes containing peaches (Schnabel and Mercier, 2006), grapes (Mercier et al., 2005), as well as cherries and raspberries (J. Mercier, unpublished data). The treatment could be applied passively by simply placing activated *M. albus* sachets within packages of grapes as is now done with sulfur dioxide generator pads. *M. albus* produces a "musky" odor that declines rapidly within packages after the sachets are removed. The level of biofumigation activity is directly affected by the storage temperature, and therefore, larger doses may be required at lower storage temperatures. As *M. albus* releases volatiles slowly at low temperatures, this technology does not provide a fast postharvest sanitation as achieved with other gases such as sulfur dioxide (SO_2) or ozone (O_3), but its continuous long-term release of active volatiles can protect the grapes during storage and transport.

Ozone is another alternative fumigant that has been tested to control postharvest decay of grape. Ozone is a natural substance in the atmosphere and one of the most potent sanitizers against a wide spectrum of microorganisms (Khadre et al., 2001). It is classified as GRAS (generally recognized as safe) for food contact applications in the USA (US Food and Drug Administration, 2001). The product of ozone degradation is oxygen; therefore it leaves no residues on treated commodities. A single fumigation with 200 $\mu L\,L^{-1}$ ozone for 4 h (Mlikota Gabler et al., 2002) or overnight fumigation with 500 $\mu L\,L^{-1}$ ozone (Shimizu et al., 1982) reduced gray mold decay in stored table grapes. A single fumigation of grapes with high concentrations (up to 10,000 $\mu L\,L^{-1} \times h$) during the pre-cooling of grapes significantly reduced gray mold in storage (Mlikota Gabler et al., 2007). Continuous fumigation during storage with a low dose of ozone (0.1–0.3 $\mu L\,L^{-1}$) for 7 weeks at 5 °C, prevented aerial mycelial growth (nesting) from *B. cinerea* among 'Thompson Seedless' grapes, but did not decrease the number of gray mold infections (Palou et al., 2002), even when used in combination with modified atmosphere packaging (Artes-Hernandez et al., 2004, 2007). As ozone does not have residual activity, it would be desirable to combine it with another treatment for more prolonged decay control.

Our objectives were to evaluate the novel integrated treatment to control postharvest gray mold of table grapes which consisted of an initial fumigation with high concentrations of ozone (5000 $\mu L\,L^{-1}$ for 1 h) during the pre-cooling phase followed by the continuous in-package biofumigation with *M. albus* during cold storage of grapes, as a way to replace sulfur dioxide fumigation. Another approach consisted of an initial fumigation of grapes with sulfur dioxide during pre-cooling followed by a continuous in-package biofumigation with *M. albus* during cold storage, as a way to replace weekly sulfur dioxide fumigation during cold storage or sulfur dioxide generator pads. Integrated treatments were evaluated in larger semi-commercial tests and compared to conventional sulfur dioxide treatments. The compatibility of *M. albus* with ozone and sulfur dioxide fumigations was evaluated by measuring volatile production and fungicidal activity of *M. albus* after exposure to those fumigants.

2. Materials and methods

2.1. Inoculum preparation

A *B. cinerea* isolate from grape (isolate 1440 obtained from T.J. Michailides, UC Kearney Agricultural Center, Parlier) was grown on potato dextrose agar (PDA) for 2 weeks at 23 °C. Spores were dislodged from the colony surface with a glass rod after the addition of a small volume of sterile water with 0.05% (wt./vol.) Triton X-100 surfactant. The spore suspension was filtered through four layers of cheesecloth and diluted with sterile water to an absorbance of 0.25 at 425 nm as determined by a spectrophotometer. This density contained 1×10^6 conidia mL^{-1} and was diluted with sterile deionized water to obtain the desired spore concentrations.

2.2. Fruit

Organically grown, freshly harvested, 'Autumn Seedless' and 'Thompson Seedless' grapes were used in experiments.

2.3. Biofumigant

M. albus formulation that consisted of rye grain colonized with *M. albus* isolate 620 as described in Mercier et al. (2007) was used in our experiments. The grain formulation was air dried at room temperature and stored at −8 °C prior to use. Optimal rates of *M. albus* were prepared in volatile-generating sachets made with heat-sealable grade 126/3 tea-bag paper (Schoeller and Hoesch N.A. Inc., Pisgah Forest, NC) and placed among the grapes. *M. albus* sachets were activated by soaking in water for 15 s.

2.4. Effect of ozone and sulfur dioxide fumigation on biofumigation activity of M. albus

The compatibility of *M. albus* with ozone or sulfur dioxide fumigation was evaluated: (i) after *M. albus* was fumigated with ozone or sulfur dioxide, the ability of its volatiles to kill *Penicillium expansum* was determined; and (ii) the production of isobutyric acid, the most abundant volatile organic compound produced by rye grain culture of *M. albus* (Mercier and Jiménez, 2007), was quantified. Isobutyric acid was previously identified as an indicator of the antimicrobial activity of the volatiles of *M. albus* (Jiménez and Mercier, 2005). The colony growth of *P. expansum* has been used by AgraQuest Inc. for evaluating the potency of *M. albus* culture batches (Mercier et al., 2007). In our experience, *P. expansum* is more resistant in vitro to the *M. albus* volatiles than *B. cinerea* (J. Mercier, unpublished data).

Activated *M. albus* sachets containing 100 g of rye grain culture were fumigated at 5 °C with 0 (control) or 5000 $\mu L\,L^{-1} \times h$ ozone. Ozone fumigation was applied within a small fumigation chamber (Tahoe Foods Technology, Inc., Sparks, NE) with internal volume of 31.58 L, Model CD12 (Clearwater Tech L.L.C., San Luis Obispo, CA), equipped with a Clearwater corona discharge ozone gas generator, and Hankin ozone gas analyzer, Model HA-100-GTP-12 (Ozocan Corp., Scarborough, Ontario, Canada). Ozone was applied at a constant concentration of 5000 $\mu L\,L^{-1}$ ozone for 1 h. The temperature during fumigation was 5 ± 2 °C. To facilitate penetration of the gas, this equipment applied ozone under moderate vacuum (33 kPa). Similarly, in a separate experiment, activated sachets were fumigated with 0 (control) or 200 $\mu L\,L^{-1} \times h$ sulfur dioxide at a commercial fumigation and storage facility. Three replicate sachets were fumigated with ozone and four replicate sachets were fumigated with sulfur dioxide, for each treatment. From each

sachet, sub-samples of 5 and 10 g of fumigated *M. albus* formulation were placed in plastic cups, rehydrated with about 0.5 mL of water per gram of *M. albus* formulation, and individually placed in closed 11.4 L plastic boxes at 20 °C. The fungicidal activity of *M. albus* was evaluated by placing two PDA *Petri* dishes freshly plated using a spiral plater with *P. expansum* suspension of 10^5 conidia mL^{-1} inside the closed box. There was no physical contact between the PDA plates and *M. albus* culture. The controls consisted of boxes without *M. albus* and boxes with non-fumigated *M. albus* culture. Plates were exposed to *M. albus* volatiles for 24 or 48 h and then *M. albus* was removed from the boxes. After *M. albus* was removed, the plates were additionally incubated until the control plate showed typical blue sporulation (about 4–5 d at room temperature) and the growth of *P. expansum* was recorded visually as a percentage of the control plates.

Volatiles produced by fumigated *M. albus* were withdrawn from closed 11.4 L plastic boxes after 24 h incubation at 20 °C. Volatiles were trapped using a solid-phase micro-extraction (SPME) syringe (Supelco): 50/30 μm DVB/Carboxen™/PDMS StableFlex™ fiber for 25 min. The syringe was then inserted into a gas chromatograph (Hewlett-Packard 5890 series II) equipped with a flame ionization detector (FID). Instrument settings as follows: injector temp: 250 °C, detector temp: 250 °C, initial oven temp: 3 °C, final oven temp: 220 °C, ramp rate: 5 °C min^{-1}, total run time: 43.80 min, carrier gas: helium (UltraHigh Purity) and column head pressure at 105 kPa. A Zebron ZB-Wax 30 m × 0.25 mm ID × 0.5 mm film thickness (liquid phase: 100% polyethylene glycol) was used to separate each component in the headspace above fungal mycelium. Prior to trapping gases, the fiber was conditioned at 250 °C for 30 min in the injector port under helium.

An alternate method was employed to analyze the volatiles produced after sulfur dioxide fumigation because our equipment was upgraded to a more automated system. Volatiles were trapped using a Gerstel Twister PDMS stir bar, 1.0 cm × 0.5 mm phase, from Gerstel Inc., Baltimore, MD. The pre-conditioned Twister stir bars were exposed to the volatile organic compounds produced by the reactivated formulated material for 25 min. After exposure, Twister stir bars were removed and placed inside the Twister desorption liners. Each liner containing a Twister bar was individually handled using a MPS2 autosampler and delivered to the thermal desorption unit (TDU) were volatiles are thermally desorbed from the PDMS phase coating the stir bars. Released volatiles are preconcentrated into a cooled injection system (CIS4) before they are sent into the GC column (Agilent 6890 equipped with FID). A Zebron ZB-Wax 30 m × 0.25 mm ID × 0.5 mm film thickness (liquid phase: 100% polyethylene glycol) was used to separate each component. Instruments settings as follows: (1) CIS4 solvent vent mode using a glass wool liner, pressure: 92 kPa, vent flow: 50 mL min^{-1}, Purge flow: 48 mL min^{-1}, CIS temperature program: (i) initial temp; −120 °C, (ii) equilibration: 0.5 min, (iii) initial time: 0.2 min, (iv) ramp: 12 °C s^{-1}, (v) end temp: 280 °C, (vi) hold time: 1.5 min. (2) TDU: (i) initial temp: 30 °C, (ii) delay time: 0.5 min, (iii) ramp: 100 °C min^{-1}, (iv) end temp: 250 °C, (v) hold time: 1.25 min. (3) GC column at constant flow (1.2 mL min^{-1}) and helium as carrier gas (UltraHigh Purity). (4) Detector temp: 250 °C. (5) Oven settings: (i) initial temp: 40 °C, (ii) initial time: 1 min, (iii) ramp 1: 10 °C min^{-1} to 125 °C and hold 0.5 min; ramp 2: 15 °C min^{-1} to 230 °C and hold 3 min. Total run time was 20 min.

2.5. Small scale experiment employing M. albus and ozone treatment, applied alone or in combination to control gray mold

'Autumn Seedless' grape clusters were divided into small clusters of approximately 100 g each and randomized so that a portion of each cluster was represented in each treatment. Initial, short-term fumigation with 5000 μL L^{-1} × h ozone during pre-cooling of the grapes and continuous biofumigation with in-package generators containing *M. albus* were evaluated to control gray mold on inoculated 'Autumn Seedless' grapes. Grapes were inoculated by briefly spraying them with 10^5 conidia mL^{-1} of *B. cinerea* 24 h prior to the initiation of the treatments. Inoculated grapes were incubated at 15 °C before treatments. Ozone fumigation was applied within a small fumigation chamber described previously. Treatments were applied separately or combined into a single application. Grapes packed in perforated cluster bags ventilated with holes in the back and front (2.7% vented area) were fumigated and then stored in covered plastic containers for 6 d at 15 °C. Treatments were: (i) untreated control; (ii) initial fumigation with 5000 μL L^{-1} ozone for 1 h; (iii) *M. albus* biofumigation (9 g sachet of rye culture per cluster bag); (iv) initial fumigation with 5000 μL L^{-1} ozone for 1 h with *M. albus* present among grapes; and (v) initial fumigation with 5000 μL L^{-1} ozone, *M. albus* was added to grapes after ozone fumigation. A total of five replicates were treated and each replicate contained 1 cluster bag with approximately 650 g of grapes. The experiment was done once.

2.6. Semi-commercial tests to evaluate and compare the effectiveness of single or combined ozone, M. albus or sulfur dioxide treatments

Organically grown 'Thompson Seedless' grapes were commercially harvested and packaged. Experiments were designed to evaluate and compare the effectiveness to control postharvest gray mold of: (i) single fumigation of grapes during pre-cooling with ozone or sulfur dioxide; (ii) continuous fumigation during storage with sulfur dioxide generator pad or *M. albus* grain formulation. Treatments were applied separately or both approaches were combined in a single treatment. The entire experiment was prepared in duplicate. In the first set, the grapes were packed in perforated cluster bags ventilated with holes in the back and front (2.7% vented area), containing approximately 1 kg of grapes, with 9 cluster bags per expanded polystyrene foam box (EPS). In the second set, the grapes were packed in clamshells with approximately 2 kg of grapes, with total of 4 clamshells per returnable plastic container (RPC). Each grape box contained approximately 8–9 kg of grapes. To evaluate the mycelial growth and berry-to-berry spread by *B. cinerea*, a grape berry with initial symptoms of gray mold, that was inoculated 3 d earlier with this pathogen, was placed in the middle of grape cluster within four corner cluster bags or within each clamshell as inoculum source.

M. albus sachets (4 × 25 g; 100 g per grape box) were placed within commercial packages at the time of harvest; they were placed beneath grape cluster bags or inside clamshell box. Treatments were: (i) Control (untreated grapes); (ii) single fumigation with 5000 μL L^{-1} × h ozone during initial pre-cooling; (iii) single fumigation with sulfur dioxide (200 μL L^{-1} × h) during initial pre-cooling; (iv) single fumigation with 5000 μL L^{-1} × h ozone during initial pre-cooling plus continuous biofumigation with in-package *M. albus* grain formulation; (v) continuous biofumigation with in-package *M. albus* grain formulation; (vi) continuous fumigation with slow release in-package sulfur dioxide generator pad (Proteku, 7 g sodium metabisulfite, Infruta, S.A., Chile); (vii) single fumigation with sulfur dioxide (200 μL L^{-1} × h) during initial pre-cooling plus weekly room fumigation with sulfur dioxide; and (viii) single fumigation with sulfur dioxide (200 μL L^{-1} × h) during initial pre-cooling plus continuous biofumigation with in-package *M. albus* grain formulation. Ozone fumigation was applied within a semi-commercial chamber with internal volume of 262.24 L, Model CD2000P (Clearwater Tech L.L.C., San Luis Obispo, CA), equipped with a Clearwater corona discharge ozone gas generator, and Hankin ozone gas analyzer, Model HA-100-GTP-12 (Ozocan Corp., Scarborough, Ontario, Canada).

After treatments were applied, the four boxes that comprised one treatment were placed on a mini-pallet and wrapped with polyethylene stretch-film with the exclusion of experiments that included weekly fumigation with sulfur dioxide during storage. The purpose of a stretch-film was to minimize rachis drying and to maximize the effectiveness of *M. albus* volatiles from rye grain formulation and sulfur dioxide gas released from sulfur dioxide generator pads. Grapes were stored in a cold room in USDA, Parlier, except treatments that had weekly sulfur dioxide fumigation, these were stored at a commercial grape storage facility.

After 30 d of storage at 0.5 °C, grapes were evaluated for gray mold incidence and grape quality: (i) Decay adjacent to inoculated berry (%) = weight of decayed berries adjacent to inoculated berry/weight of grapes in 4 cluster bags or clamshells that contained inoculated berry × 100; (ii) Spread of aerial mycelium from inoculated berry (cm^2) = average mycelia surface in 4 cluster bags or clamshells that contained inoculated berry; (iii) Natural gray mold (%) = weight of decayed berries other than around inoculated berry/weight of cluster bags or clamshells × 100; (iv) Rachis condition (1–5 scale; where 1 = whole rachis and pedicels green and fresh; 2 = primary and secondary rachis green, pedicels brown; 3 = primary rachis green, secondary rachis brown; 4 = primary rachis 50% brown, secondary rachis brown; 5 = whole rachis brown and brittle).

2.7. Statistical analyses

Homogeneity of variances was determined using Levene's test. The incidence of gray mold was analyzed by ANOVA applied to the arcsin of the square root of the proportion of infected berries (SPSS 15.0, SPSS Inc., Chicago, IL). Means were separated by Fisher's protected least significant difference test ($P \leq 0.05$). Actual values are shown.

3. Results

3.1. Effect of ozone and sulfur dioxide fumigation on survival and biofumigation activity of M. albus

M. albus survived fumigation with either ozone or sulfur dioxide; these treatments did not affect the antifungal activity of the rye formulation of *M. albus* that was measured by the inhibition of *P. expansum* colony growth. Both ozone- or sulfur dioxide-fumigated *M. albus* grain formulation completely killed *P. expansum*, when freshly plated PDA cultures were exposed to 5 or 10 g of rye culture for 48 h in closed 11-L plastic boxes, resulting in clear plates with no sign of fungal growth (data not shown). The ozone fumigation did not affect the production of isobutyric acid by *M. albus* (Table 1), while the sulfur dioxide fumigation reduced the production of isobutyric acid, suggesting that this treatment might be detrimental to *M. albus*.

Table 1
Effect of ozone (O_3) or sulfur dioxide (SO_2) fumigation on the activity of a rye grain formulation of *Muscodor albus*. Sachets containing 100 g of activated *M. albus* formulation were fumigated with 5000 $\mu L L^{-1}$ × h ozone or 200 $\mu L L^{-1}$ × h sulfur dioxide. *M. albus* formulation activity was evaluated by measuring isobutyric acid (IBA) production ($\mu g L^{-1}$ ± SD) after incubation of the *M. albus* formulation in 11.4 L boxes for 24 h at 20 °C.

Fumigant	Formulation (g/box)	IBA production in 11.4 L boxes	
		Non-fumigated control	Fumigated
O_3	5	19.55 ± 0.58	18.46 ± 0.83
SO_2	5	13.60 ± 1.75	7.45 ± 4.21
SO_2	10	15.70 ± 1.24	11.24 ± 1.24

Table 2
Gray mold incidence and rachis appearance of 'Autumn Seedless' grapes. Ozone (O_3) fumigation was combined with *M. albus* biofumigation. Ozone fumigation consisted of 5000 $\mu L L^{-1}$ applied for 60 min. *M. albus* was applied by the addition of one 9 g teabag containing 9 g of *M. albus* grain formulation per grape cluster bag. Grapes were treated in perforated cluster bags and stored in covered plastic containers for 6 d at 15 °C. One replicate was a cluster bag containing approximately 650 g of grapes, with a total of 5 replicates per treatment. Grapes were inoculated by spraying them with *Botrytis cinerea* conidia 24 h prior to treatments. The inoculated grapes were kept in humid boxes at 15 °C until treated. Treatments followed by unlike letters differ significantly at $P \leq 0.05$, according to Fisher's Protected LSD.

Treatments	Gray mold (%)	Rachis (1–5)[a]
Untreated control	91.7 a	2.2 a
O_3	19.3 b	2.3 a
M. albus	21.2 b	2.2 a
O_3 + *M. albus*	10.1 c	2.3 a
O_3 followed by *M. albus*	9.5 c	2.5 a

[a] Visual index where 1 = green and fresh, 5 = brown and brittle.

3.2. M. albus and ozone treatments, applied alone or in combination to control gray mold

Gray mold incidence among inoculated 'Autumn Seedless' grapes was reduced from 91.7 (untreated) to 19.3% after a single fumigation of grapes packed in cluster bags with 5000 $\mu L L^{-1}$ ozone, and further to 10% when ozone and *M. albus* were combined (Table 2).

In semi-commercial experiments with 'Thompson Seedless' grapes, where the efficacy of ozone, *M. albus*, and sulfur dioxide treatments were compared to control gray mold, ozone and *M. albus* were less effective than sulfur dioxide treatments (Figs. 1A, 2A, 2B). Initial fumigations with sulfur dioxide or ozone, alone or combined with *M. albus*, did not control mycelial growth from an inoculated berry (Fig. 1B), while treatment with sulfur dioxide generator pads or with initial and weekly sulfur dioxide fumigation inhibited it effectively.

The addition of *M. albus* to grape package before pre-cooling with sulfur dioxide did not result in additional reduction in gray mold incidence or spread among berries (Figs. 1A, 1B, 2A, 2B). Treatment with sulfur dioxide generator pads, where grapes were pre-cooled with air, was equally effective as treatment with weekly sulfur dioxide fumigation, where grapes were pre-cooled with sulfur dioxide gas (Figs. 1A, 1B, 2A, 2B). Overall, there was less gray mold, either naturally occurring or adjacent to inoculated berry, among grapes packaged in clamshell/RPC, compared to cluster bag/EPS packaging (Fig. 2A and B).

Natural gray mold incidence among 'Thompson Seedless' grapes was significantly reduced when ozone and *M. albus* were combined, compared to when applied alone (Figs. 1A and 2A). Natural decay was reduced from 9.7 when grapes were fumigated with ozone to 3.4 when *M. albus* was added to EPS boxes prior to ozone fumigation (Fig. 2A). Natural decay was reduced from 3.4 when grapes were fumigated with ozone to 1.9 when *M. albus* was added to RPC boxes prior to ozone fumigation (Fig. 2A).

Ozone alone or when combined with *M. albus* in a single treatment did not control mycelial growth from inoculated berry (Fig. 1B), while treatment with sulfur dioxide generator pad and treatment with initial and weekly sulfur dioxide fumigation inhibited mycelial growth. Within both types of packaging, gray mold spread from previously inoculated berries inside the grape cluster was not successfully controlled by an initial ozone fumigation, but was suppressed by continuous biofumigation with *M. albus* (Fig. 2B). The efficacy of a sulfur dioxide generator pad in controlling natural and gray mold that spread from inoculated berry was equal to the treatment where grapes were pre-cooled and weekly fumigated with sulfur dioxide gas (Fig. 2A and B).

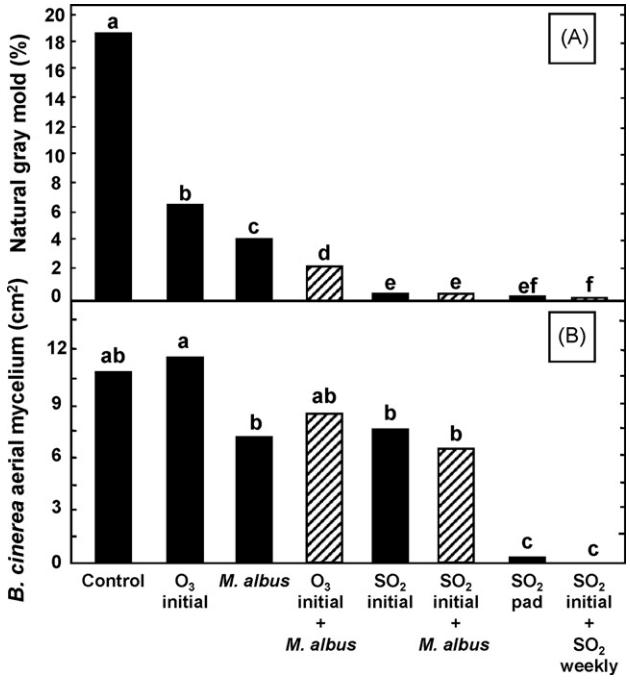

Fig. 1. Natural gray mold incidence (A) and *Botrytis cinerea* aerial mycelium spread from previously inoculated berry (B) among commercially packed 'Thompson Seedless' table grapes after 30 d storage at 1 °C. Treatments were applied alone (solid columns) or in combinations (striped columns): (i) Control (untreated); (ii) single fumigation with ozone (O_3, 5000 μL L^{-1} × h) during initial pre-cooling; (iii) single fumigation with sulfur dioxide (SO_2, 200 μL L^{-1} × h) during initial pre-cooling; (iv) single fumigation with ozone (5000 μL L^{-1} × h) during initial pre-cooling plus continuous biofumigation with in-package *Muscodor albus* grain formulation; (v) continuous biofumigation with in-package *M. albus* grain formulation; (vi) continuous fumigation with slow release in-package sulfur dioxide generator pad (7 g sodium metabisulfite); (vii) single fumigation with sulfur dioxide (200 μL L^{-1} × h) during initial pre-cooling plus weekly room fumigation with sulfur dioxide; and (viii) single fumigation with sulfur dioxide (200 μL L^{-1} × h) during initial pre-cooling plus continuous biofumigation with in-package *M. albus* grain formulation. Values are means of two experiments. There were four replicates per treatment in each experiment. In the first experiment, fruit were packed in cluster bags and placed in expanded polystyrene foam (EPS) boxes; in the second experiment, they were packed in clamshell boxes and placed in returnable plastic containers (RPC). Columns with unlike letters differ significantly ($P \leq 0.05$) according to Fisher's Protected LSD test.

3.3. Grape quality evaluation

The appearance of berries was not harmed by any of the treatments. Rachis appearance was most harmed after treatments with ozone, followed by treatments that included pre-cooling with sulfur dioxide (Fig. 2C). The rachis condition in grape packages that contained *M. albus* and sulfur dioxide generator pads that were not pre-cooled with sulfur dioxide gas was better than among those in treatments where the grapes were pre-cooled with sulfur dioxide gas. The rachis of grapes fumigated with ozone sometimes developed thin longitudinal darkened lesions.

4. Discussion

Control of decay among table grapes caused by naturally occurring inoculum on the berry surface (Fig. 2A) was improved by combining an initial ozone fumigation with continuous in-package fumigation with *M. albus*. Ozone provided fast and effective initial sanitation of grapes and reduced the viable inoculum on grapes, while *M. albus* continued to suppress gray mold that developed from infections that were protected within the plant tissue that ozone could not kill. In experiments where 'Autumn Seedless' grapes were spray-inoculated with *B. cinerea* conidia 24 h before treatment and stored at room temperature, gray mold incidence was lower when ozone and *M. albus* were combined, than if either was applied alone.

Treatments that included sulfur dioxide fumigation were more effective in controlling postharvest gray mold than ozone or *M. albus*. Microorganisms embedded in fruit tissues are more resistant to ozone than those on fruit surfaces (Mahapatra et al., 2005). In solution, sulfur dioxide diffuses through membranes and accumulates in microorganisms by an ionization–entrapment mechanism (Smilanick et al., 1990a). The epicuticular wax of grape berries minimizes penetration of the gas and allows them to tolerate sulfur dioxide fumigation without injury. Its penetration into berries is modest (a brief, reversible loss of berry color occurs) and its residues persist typically less than 24 h (Smilanick et al., 1990b). It can accumulate to high levels and persist in injured berries, pedicels, and the rachis, where it causes bleaching injuries (Smilanick et al., 1990b). The penetration of sulfur dioxide into pedicels may be particularly important, since the majority of berry infections with *B. cinerea* infections occur at pedicel-berry attachment zone (Michailides et al., 2000; Holz et al., 2003). Many berry infections originate from flower part infections during bloom where the pathogen persists inconspicuously as an endophyte, and it emerges later to cause postharvest gray mold (Elmer and Michailides, 2004).

Within both types of packaging (cluster bags in EPS boxes or clamshells in RPC), the gray mold infections that originated from previously inoculated berry that was placed inside grape clusters before treatments were not successfully controlled by initial ozone fumigation (Fig. 2B), but they were suppressed by *M. albus* continuous fumigation. There was less gray mold, either naturally occurring or adjacent to inoculated berries, among grapes in clamshell/RPC packaging, compared to those in cluster bag/EPS packaging. The rigid clamshell container protected the berries from mechanical injuries during the packaging process, thus making them less susceptible to subsequent infections.

The integration of ozone and *M. albus* is easily achievable because of the negligible effect of the initial ozone fumigation on the biological activity of the *M. albus* sachets. The combined use of ozone and *M. albus* in grapes could be feasible because it is compatible with the various phases of the handling process, packaging, and storage within export containers, or within the fruit packages themselves. Since *M. albus* was not affected by a single ozone fumigation, the treatment could be applied passively by simply placing the activated *M. albus* pad inside the package, as is done with sulfur dioxide generator pads, and followed by fumigation with ozone during pre-cooling of the table grapes. Both sulfur dioxide pads and *M. albus* treatments should be used with packaging that will enable the containment of released gas or volatiles, such as the perforated box liners or the external pallet wrapping used in our experiments.

A single sulfur dioxide fumigation did not kill *M. albus* within the grain formulation, although it reduced the production of isobutyric acid, which is an indicator of volatile antifungal activity for this isolate. This suggests that it might be possible to integrate sulfur dioxide and *M. albus* biofumigation in the storage of table grapes, with the possibility of reducing the rate or the number of sulfur dioxide fumigations during conventional storage of grapes. The detrimental effect of sulfur dioxide on *M. albus* activity could be alleviated by increasing the dose of biofumigant used, in order to compensate for the reduction in volatile production caused by sulfur dioxide fumigation. Because of the high level of effectiveness of the initial sulfur dioxide fumigation during forced-air pre-cooling of grapes in our experiments, we could not quantify the contribution of *M. albus* to further reduce gray mold.

An issue with the use of *M. albus* is that it is alive and its metabolism and efficacy depend on the rye grain substrate on which it is grown. *M. albus* requires at least 2 h at ambient temperature after rehydration to properly reactivate for use at cold

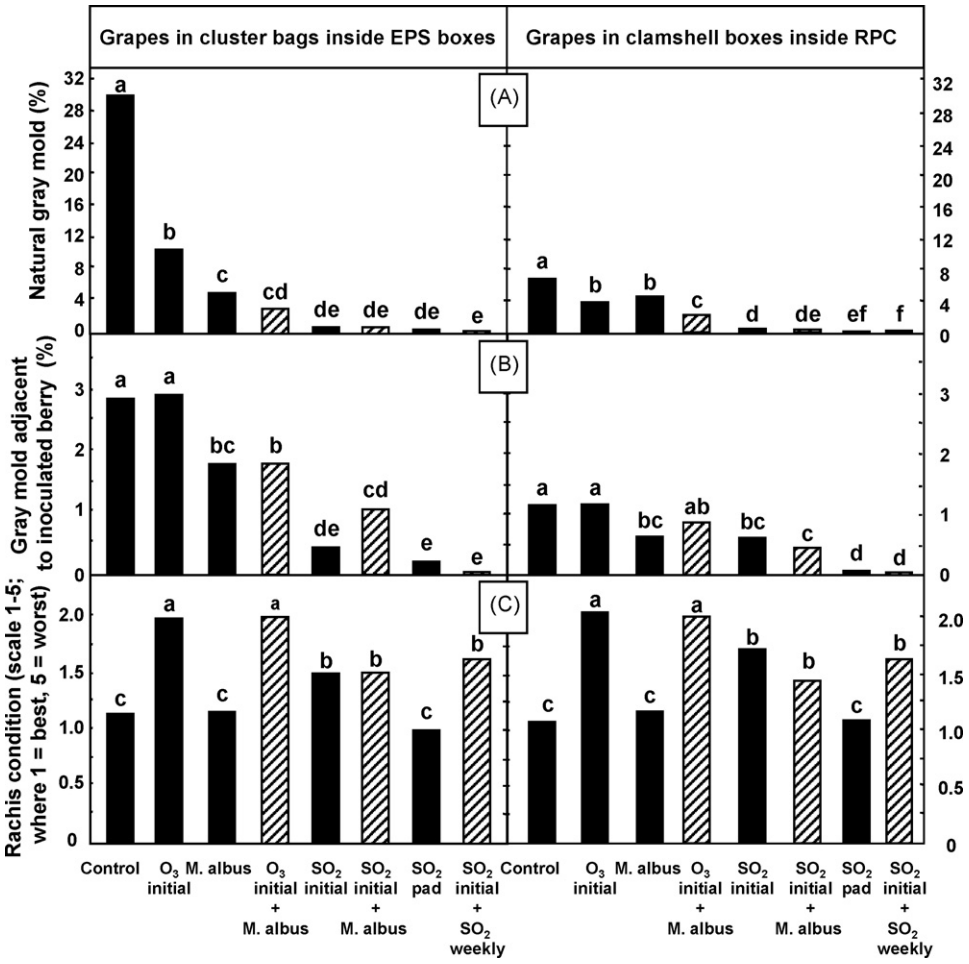

Fig. 2. Postharvest gray mold and rachis appearance of 'Thompson Seedless' table grapes after various treatments and 30 d of storage at 0.5 °C. (A) Gray mold that originated from natural infections. (B) Gray mold that spread to adjacent berries from an initial berry that was artificially inoculated 3 d before the treatments were applied and placed among clusters in four cluster bags or clamshells during the packaging of grapes. (C) Rachis appearance (1–5 scale; where 1 = green and fresh; to 5 = brown and brittle). Treatments were applied alone (solid columns) or in combinations (striped columns): (i) Control (untreated); (ii) single fumigation with ozone (O_3, 5000 $\mu L\,L^{-1} \times h$) during initial pre-cooling; (iii) single fumigation with sulfur dioxide (SO_2, 200 $\mu L\,L^{-1} \times h$) during initial pre-cooling; (iv) single fumigation with ozone (5000 $\mu L\,L^{-1} \times h$) during initial pre-cooling plus continuous biofumigation with in-package *M. albus* grain formulation; (v) continuous biofumigation with in-package *M. albus* grain formulation; (vi) continuous fumigation with slow release in-package sulfur dioxide generator pad (7 g sodium metabisulfite); (vii) single fumigation with sulfur dioxide (200 $\mu L\,L^{-1} \times h$) during initial pre-cooling plus weekly room fumigation with sulfur dioxide; and (viii) single fumigation with sulfur dioxide (200 $\mu L\,L^{-1} \times h$) during initial pre-cooling plus continuous biofumigation with in-package *M. albus* grain formulation. Each value is the mean of four replicates per treatment per each package type. Fruit was packed in cluster bags in expanded polystyrene foam (EPS) boxes or in clamshell containers in returnable plastic containers (RPC). Within each package type, columns with unlike letters differ significantly ($P \leq 0.05$) according to Fisher's Protected LSD test.

temperatures (Jiménez and Mercier, 2005). In the case of vineyard-packed grapes, this reactivation time would elapse when the grape boxes are collected in the field and brought to the storage facility for pre-cooling. Because there is detectable volatile production within a few hours at ambient temperature (Mercier and Jiménez, 2007), it is possible that some biofumigation activity can take place before the grapes are pre-cooled. However, time in the field can be variable and volatiles produced before cold storage would be ineffective unless the sachets and fruit are placed in containers that can prevent their escape. *M. albus* activity is also temperature dependent; not only are volatiles produced faster at ambient temperature than at 0.5 °C, fungi are usually killed faster at warmer temperatures than at low temperatures (J. Mercier, unpublished data). For example, reactivated *M. albus* rye grain formulation at a rate of 0.9 g L^{-1} of air completely killed *Penicillium expansum* conidia and controlled blue mold of apple within 24–48 h at ambient temperature, while it took about 3 weeks to achieve the same results at 4 °C. *M. albus* more effectively controlled postharvest gray mold on grapes at ambient temperatures than at cold temperatures (Mlikota Gabler et al., 2006). However, *M. albus* can protect grapes for several weeks in cold storage and its biofumigation activity would likely continue during shipping, conferring long-term protection of the grapes.

The use of stretch-film to wrap pallets of pre-cooled grapes was a convenient method to contain sulfur dioxide gas from generator pads (Lichter et al., 2008) or volatiles produced by *M. albus* within the grape packages and minimize their escape. Sulfur dioxide pads and *M. albus* require the use of box liners in order to contain the gases within the package. When used in previous experiments in EPS boxes without liner (Mercier et al., 2005), considerable escape of *M. albus* volatiles could have taken place through the holes in EPS boxes, which are designed to allow the penetration of sulfur dioxide during weekly fumigations. The escape of the volatiles likely reduced the efficacy of the biofumigation process (Mercier et al., 2005). Such rate of volatile escape is likely to be affected by how tightly the EPS boxes are packed and whether holes are being obstructed by the fruit in tightly packed boxes. Liners are perforated to facilitate the penetration of air during forced-air pre-cooling and, depending on the area perforated, can significantly prolong the time required to pre-cool grapes (Cantin and Crisosto,

2008). By wrapping the pallets externally with stretch-film after the grapes have been pre-cooled, we avoided increased cooling times, while providing a suitable package for sulfur dioxide generator pads or *M. albus*. Stretch-film minimized rachis drying, stabilized the boxes when arranged in pallets, and maximized the effectiveness of *M. albus* volatiles and sulfur dioxide released from generator pads. This method, used in conjunction with *M. albus* or other in-package fumigants, may merit more research as a method for the commercial storage of table grapes.

Fumigation with high doses of ozone gas during pre-cooling of grapes as well as continuous fumigation of grapes with *M. albus* during storage controlled postharvest decay. When these treatments were integrated together, their effectiveness improved, but was still inferior to sulfur dioxide. These are unlikely to replace sulfur dioxide treatments in conventional grape production unless their efficacy is improved. It is possible that modifying the containers to better contain the volatiles, by the addition of liner or covering the pallets with plastic films, could help increase volatile concentration and improve the efficacy of the biofumigation process. Biofumigation might be especially interesting for use on certified organic grapes, where the use of sulfur dioxide is prohibited, or if sulfur dioxide use is discontinued for some reason.

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