



Ozone for control of post-harvest decay of table grapes caused by *Rhizopus stolonifer*

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The effect of ozone on post-harvest decay of table grapes was studied both with regard to its effectiveness and its possible mode of action. Ozone concentrations fell rapidly upon contact with organic matter and the amount which reacted with grape berries and the microflora on their surface was about 0.1 mg g⁻¹, when supplied at a rate of 8 mg min⁻¹ for 20 min. The dose applied could be increased by longer periods of exposure, but symptoms of toxicity were observed on certain cultivars. The number of colony forming units (cfu) of fungi, yeasts and bacteria naturally present on the berry surface was considerably reduced by a 20 min exposure to ozone. Ozone treatments significantly reduced the extent of berry decay caused by fungi following cold storage and increased shelf-life. A significant decrease in decay was observed in berries that were treated with ozone either before or after being inoculated with *Rhizopus stolonifer*. This finding indicates that, in addition to its sterilizing effect, ozone also induced resistance to post-harvest decay development. The phytoalexins resveratrol and pterostilbene were elicited by ozone treatments, at levels similar to those produced by uv-c irradiation. Resveratrol accumulated in greater quantities than pterostilbene. Inoculation with *R. stolonifer* in addition to ozone treatment, raised the levels of both stilbenes even more. Exposing berries to ozone was almost as effective as SO₂ fumigation for the control of storage decay caused by *R. stolonifer* and no deleterious effects were observed on the appearance of the grape cluster. Ozone treatments can therefore be considered as a possible substitute for SO₂ fumigation for the control of post-harvest fungal decay.

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INTRODUCTION

In recent years the demand to extend the post-harvest life of grape berries in Israel has increased, due to an increase in grape exports and the need to regulate the supply to the Israeli local market. However, with longer storage periods, the fruit has been prone to decay, by moulds indigenous to the vineyard, the most common one being *Rhizopus stolonifer* [3, 39].

The recommended method of controlling post-harvest grape decay is SO₂ fumigation in combination with rapid precooling, followed by maintaining a temperature of 0 °C throughout storage and marketing [6, 22, 29]. Sulphur dioxide is applied either by the gradual, continuous release of the gas from meta-bisulphite-impregnated paper or by

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repeated fumigation, by periodically releasing the gas from cylinders [6, 22, 29]. Unfortunately, SO₂ fumes are allergenic for part of the population [2, 5, 28, 36] and hence its use has been banned, or restricted to certain commodities, and even then in limited amounts. It is permitted for use with table grapes, but the maximal residual level allowed has been reduced [13]. The need to develop alternative methods is, therefore, of importance to the grape industry. Ozone is a potent antimicrobial agent and is widely used to disinfect water. However, as an air pollutant it has been shown to predispose plants to infection by *Botrytis cinerea*, probably as a result of phytotoxic effects [25, 42]. Nonetheless, at appropriate concentrations, ozone may induce resistance of plants to pathogens [18, 24, 34]. Possible biochemical reactions of plants to ozone are the production of PR proteins [12, 20, 38], induction of ethylene [27] or elicitation of phytoalexins, such as the stilbenes [32]. Since the phytoalexins found in grapes are the stilbenes resveratrol, pterostilbene and several viniferins [23], it was considered possible that ozone could be used to induce resistance to post-harvest decay in grapes.

In the present work, we examined the effects of exposing grapes to ozone on the control of post-harvest fungal decay, and present evidence to show that this is achieved as a result of both surface sterilization and elicitation of stilbene phytoalexins.

MATERIALS AND METHODS

Plant material

The table grapes (*Vitis vinifera* L.) used throughout these studies were harvested from commercial vineyards in Israel and brought to the laboratory immediately after harvest. Cultivar Perlette was harvested in July 1993, cvs. Thompson Seedless and Zeiny in September 1993, and cvs. Alphonse Lavallée and Barlinka in November 1993.

Fungal inoculations

Rhizopus stolonifer was isolated from diseased berries, and established as a single spore culture on potato dextrose agar (PDA) [21], and maintained at 4 °C. To prepare spore suspensions, the fungus was grown for 3 days in two Petri dishes containing PDA. The spores were gently removed from the cultures with 10 ml sterile distilled water per dish. The suspension was transferred to a sterile flask and the final spore concentration was adjusted to 10⁷ ml⁻¹. (This high spore concentration was used to ensure infection of the fruit without wounding). The grapes were then inoculated by immersing berries detached from clusters with their pedicels, in the suspension for 1 min. After inoculation, each berry was placed in an isolated well of a sterile polystyrene plate (Corning Cell Wells; Corning, NY, U.S.A.) and incubated at 28 °C in a saturated atmosphere. The percentage of infected berries was recorded after 72 h.

Ozone treatments in small-scale trials

Approximately 2 kg of grape clusters of each cultivar, were exposed to ozone for different periods of time (up to 30 min), in a 30 litre screw-capped Plexiglas cylinder, adapted with an entrance port for ozone application and an exit port for air exclusion. Throughout the experiment, the sealed cylinder was held on a laminar flow bench to

minimize environmental contamination. Ozone was supplied at a rate of 8 mg min^{-1} in an air flow of 500 ml min^{-1} , from an ozone generator (Ozontech, Haifa, Israel). The amount of ozone supplied was monitored with the aid of a spectrophotometer adjusted to 254 nm and fed with a bypass pipe from the entrance to the cylinder. The readings were calibrated by comparison with the values obtained by iodometric titration. The values obtained were constant throughout the experiments. The air expelled from the cylinder was captured in a flask containing 400 ml of 20 g l^{-1} KI solution and the amount of trapped ozone was determined by the iodometric titration method [4]. The difference between the levels of ingressing and egressing ozone was calculated and defined as the amount that had reacted with the grapes and with the microflora on the grape surface. Control measurements, without fruit, indicated that the amount of spontaneous ozone degradation was negligible under the experimental conditions. After treatments, clusters were transferred to sealed polyethylene bags and stored as described below.

Effects of ozone on the microflora present on berry surfaces

The effect of ozone treatment on fungi, yeasts and bacteria (as cfu) was determined after different periods of time. Following ozone treatment, five berries were detached from different clusters, immersed in sterile distilled water and shaken for 30 min. For each treatment, three 20 μl samples were sampled from the aqueous suspensions. Each sample was spread over selective media [8]. The inoculated Petri dishes were incubated at $28 \text{ }^\circ\text{C}$ for 24 h and the number of colonies was recorded.

Effect of ozone on grape decay, with or without inoculation with Rhizopus stolonifer

Grapes were treated with ozone and inoculated with *R. stolonifer* as described above. Four categories of treatment were used: (i) clusters brought to the laboratory immediately after harvest were submitted to ozone treatment for different periods of time (up to 80 min); (ii) as (i), but ozone was applied after immersing the clusters in 70% ethyl alcohol; (iii) as (i), but before ozone treatment the clusters were inoculated by immersion in a *R. stolonifer* spore suspension of 10^9 ml^{-1} ; and (iv) as in (i), followed by inoculation as in (iii). After treatment, grape clusters were incubated at $20 \text{ }^\circ\text{C}$ and 85–90% RH. Extractions for phytoalexin determination were performed 24 h after the termination of ozone treatments. The number of berries showing decay was recorded after 6 days' incubation.

Phytoalexin determination

Uniform-sized berries weighing 20 g were ground with 35 ml of methanol for 1 min in an Ultra-Turrax homogenizer kept on ice. The slurry was centrifuged for 10 min at 10000 g in a Sorval refrigerated centrifuge.

The clear supernatant was evaporated to dryness in a rotary evaporator under vacuum at $40 \text{ }^\circ\text{C}$. The residue was dissolved in 5 ml of 3% NaHCO_3 , filtered through Whatman 1MM filter paper with vacuum, and the filtrate, containing the stilbenes, was extracted four times with 10 ml ethyl acetate. The ethyl acetate fraction was then washed with 20 ml of deionized water. Aqueous residues in the organic fraction were removed by the addition of 5 g of anhydrous MgSO_4 followed by filtration. The ethyl

acetate fraction was evaporated to dryness in a rotary evaporator at 40 °C and the residue resuspended in a small volume of ethyl acetate. The mixture was placed in a 4 ml glass vial, and evaporated to dryness under nitrogen at room temperature. The residue in the vials was stored at -18 °C until used. Samples to be examined were dissolved in 500 µl of the mobile phase, filtered through DynaGard 3.9 cm² Nonsterile Syringe Filter 0.45 µm, and 15 µl was injected into an HPLC apparatus. Resveratrol and pterostilbene were separated on a Whatman Partisil PXS 10/25 column, using 4% methanol in 96% methylene chloride as the mobile phase, at a flow rate of 1 ml min⁻¹ and detected by measuring absorbance at 300 nm. The eluate was monitored with a diode array detector from 200 to 400 nm. The retention times for pterostilbene and resveratrol were 2.9 and 9.2 min, respectively. Quantitation was by comparison with standard curves prepared from purified stilbenes, kindly supplied by R. Pezet (Changins Station Fédérale de Recherches Agronomiques, Nyon, Switzerland).

RESULTS

Ozone degradation during exposure of grapes

Increasing ozone doses were achieved by extending the exposure time. The amount of ozone reacting with the grapes and the microorganisms on their surface was approximately 0.1 mg g⁻¹ grapes during 20 min of exposure (Fig. 1). This level was maintained with little change for an additional 20 min. Increasing the exposure time up to 80 min caused a linear increase in reacted ozone.

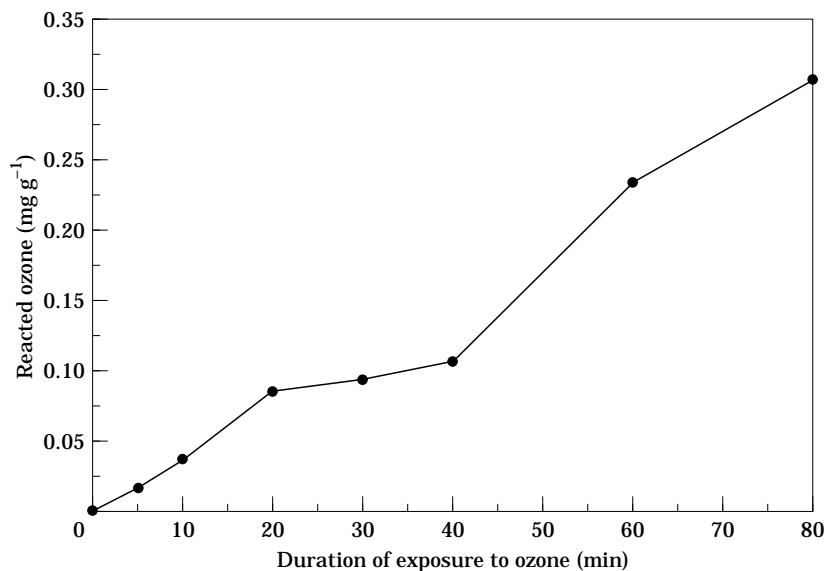


FIG. 1. Time course for ozone reaction with grape clusters and the microflora naturally present on their surface. (The data were calculated from the difference between the amount of ingressing and egressing ozone in relation to the quantity of fruit in the treatment chamber).

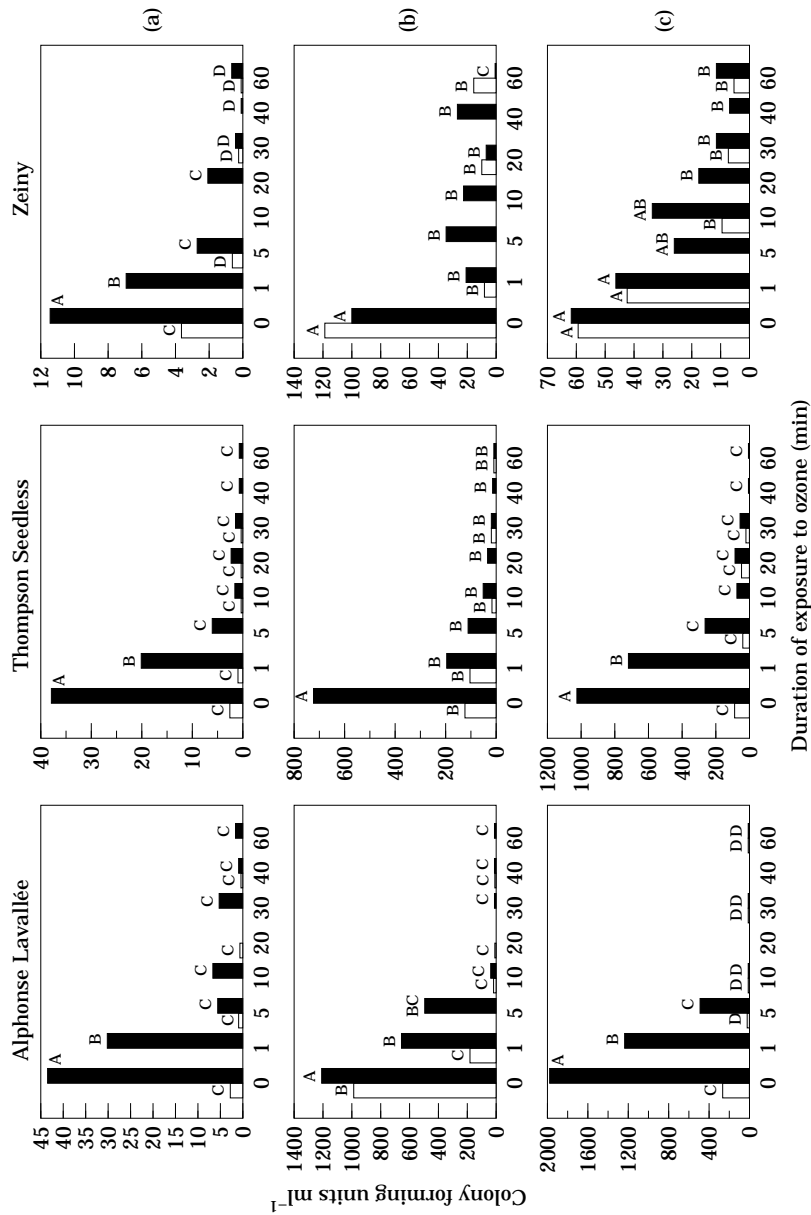


Fig. 2. Colony forming units of (a) fungi, (b) yeasts and (c) bacteria on berries of cvs. Alphonse Lavallée, Thompson Seedless and Zeiny after exposure to ozone for different periods of time. Berries were immersed (□) or not (■) in 70% ethanol prior to ozone treatments. (Assessments made after ozone exposure). Columns with different letters in each figure indicate significant differences by Duncan's multiple range test ($P \leq 0.05$).

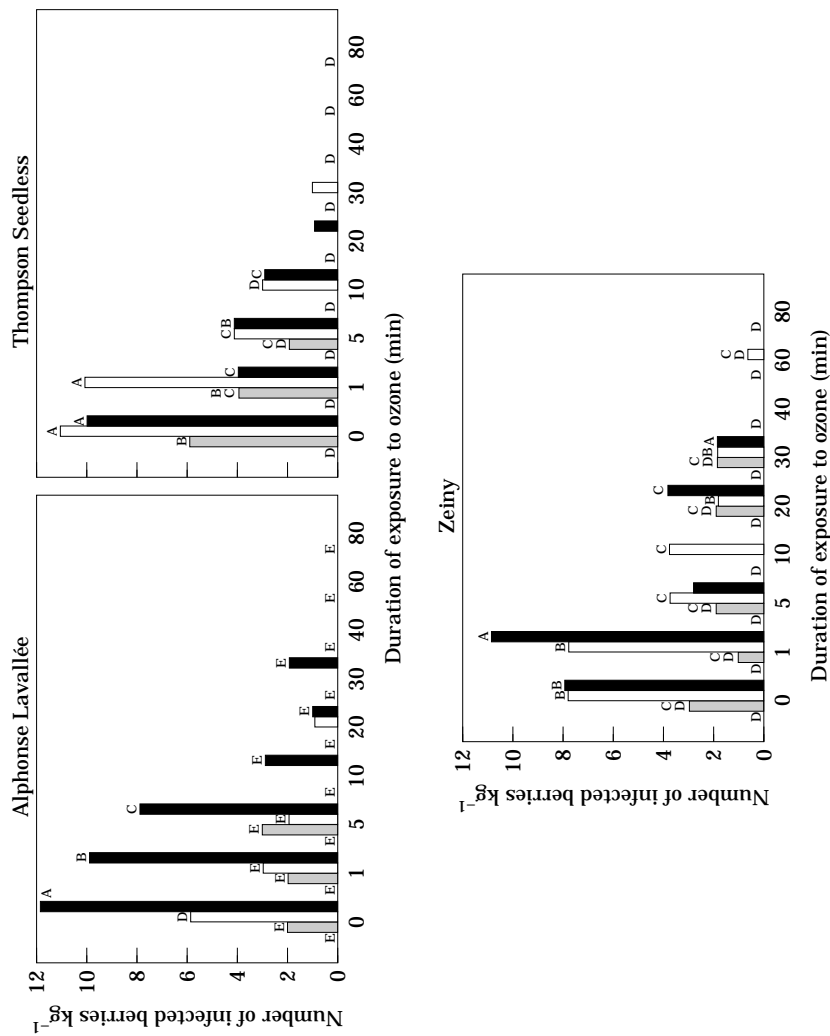


Fig. 3. Decay severity caused by fungi on berries of cvs. Alphonse Lavallée, Thompson Seedless and Zeiny, treated with 70% ethyl alcohol prior to ozone exposure (■); exposed only to ozone (□); inoculated with *Rhizopus stolonifer* before (□) and after (■) exposure to ozone for different periods of time. (Decay assessment made after 6 days at 20 °C). Columns with different letters in each figure indicate significant differences by Duncan's multiple range test ($P \leq 0.05$).

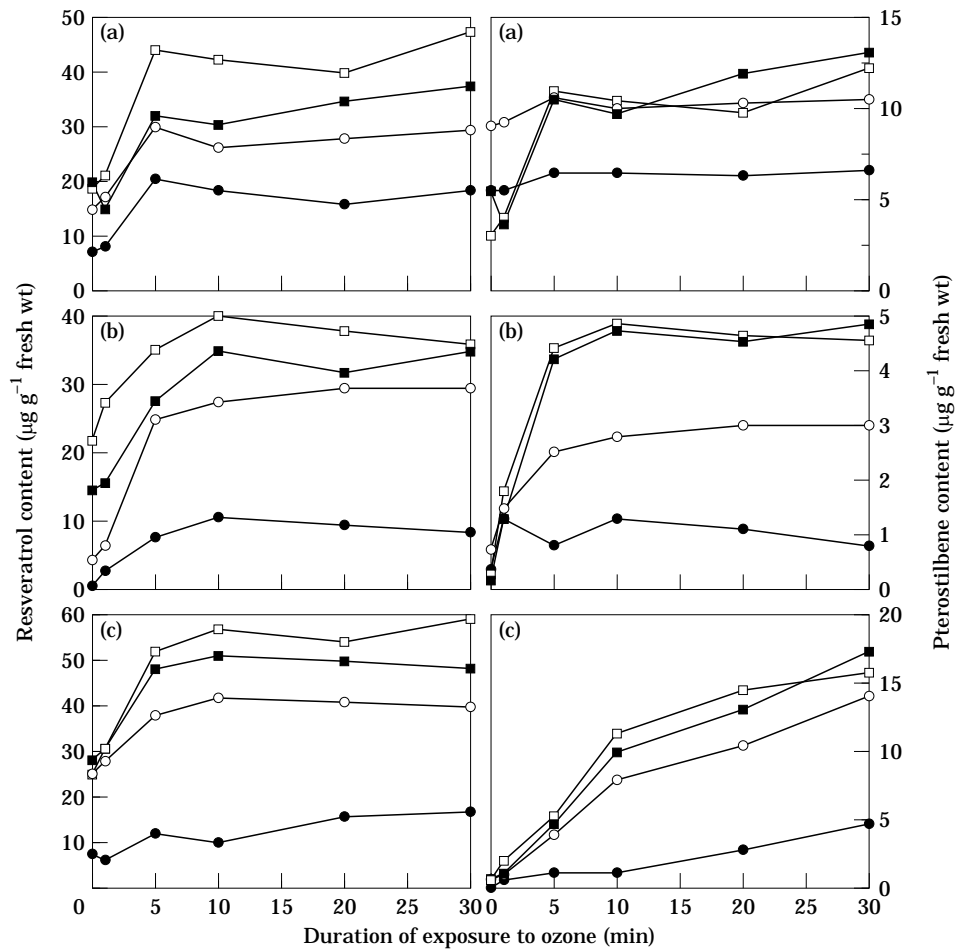


FIG. 4. Elicitation of resveratrol and pterostilbene in ripe grape berries treated with 70% ethyl alcohol prior to ozone exposure (●); exposed only to ozone (○); inoculated with *R. stolonifer* before (■) and after (□) exposure to ozone for different periods of time. Cultivars (a) Alphonse Lavallée, (b) Thompson Seedless and (c) Zeiny. The standard errors for resveratrol and pterostilbene were ± 3.437 and ± 1.167 , respectively.

Ozone phytotoxicity on freshly harvested grapes

No phytotoxicity was observed on berries, stems or pedicels of either green or red cultivars following ozone exposures lasting up to 40 min. However, after longer periods of time, increasingly severe phytotoxicity was observed in berries of cvs. Zeiny and Alphonse Lavallée. Toxicity was characterized by microscopic vein-like cracks on the epidermis (data not shown). Therefore, the level of reacted ozone obtained during 40 min, was used throughout our experiments. Firmness of the berries of all cultivars was not adversely affected by ozone treatments, even after 80 min of exposure (data not shown).

TABLE 1
 Comparison between effects of ozone and SO₂ on decay and appearance of grapes (*cv. Barlinka*) after storage at 0 °C for 5, 7 or 9 weeks followed by 3 days at 20 °C (shelf life)

Treatment	Freshness											
	Decay severity* (weeks in cold storage)			Fruit† (weeks in cold storage)			Stems‡ (weeks in cold storage)			Pedicels‡ (weeks in cold storage)		
	5	7	9	5	7	9	5	7	9	5	7	9
Control	3.75a§	4.50a	3.75a	4.75a	4.00a	4.00a	3.50a	3.75a	3.75a	4.50a	4.75a	4.50a
Ozone	2.10b	1.70b	1.90b	3.50b	3.00b	2.80b	2.80a	3.10ab	2.90b	4.10a	3.90b	3.60b
SO ₂	1.40c	1.20b	1.20b	2.10c	2.40c	2.50b	1.60b	2.50b	2.70b	2.30b	3.10c	3.60b

* 1-denotes no decay and 5 = more than 20 decayed berries per cluster

† 1-denotes fresh and 5 = poor appearance.

‡ 1-denotes green and 5 = dry appearance.

§ Within columns, numbers followed by a common letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$). Means of four replicates.

Effects of ozone on the microflora naturally present on berry surfaces

Ozone significantly reduced the microfloral population on berries of all three cultivars after exposure for 1 min only (Fig. 2). Total elimination of all three groups (fungi, yeasts and bacteria) was achieved by 20 and 40 min exposure of cvs. Alphonse Lavallée and Thompson Seedless, respectively. The microflora on *cv. Zeiny*, especially the bacteria, were less affected by ozone treatment. Immersing berries in 70% ethanol was generally as effective in reducing the microflora as a 5 min exposure to ozone, with the exception of yeasts and bacteria in *cv. Zeiny*. In the latter cases, a combination of ethanol immersion and 20 min exposure to ozone almost eliminated the whole microfloral population.

Effects of ozone on berry decay after storage and simulated shelf-life

In three of the four treatments described in Materials and Methods, exposure to ozone significantly decreased the number of berries susceptible to the fungi (Fig. 3). On fruit immersed in ethanol, no decay developed during 6 days at 20 °C, even without exposure to ozone. Thirty to forty minutes of exposure was sufficient to eliminate all decay, even in treatments where berries were heavily inoculated with *Rhizopus*.

Ozone-elicited phytoalexins

Exposure of ripe grape berries to a stream of air containing ozone, elicited the production of resveratrol and pterostilbene, the former accumulating in larger amounts than the latter. Production of both phytoalexins generally reached a maximum level 24 h after 5–10 min exposure to ozone, depending on the cultivar, and was enhanced by inoculation with *R. stolonifer*, either before or after ozone treatments (Fig. 4a and b). Ethanol treatment, on the other hand, reduced the elicitation potential of ozone for both phytoalexins.

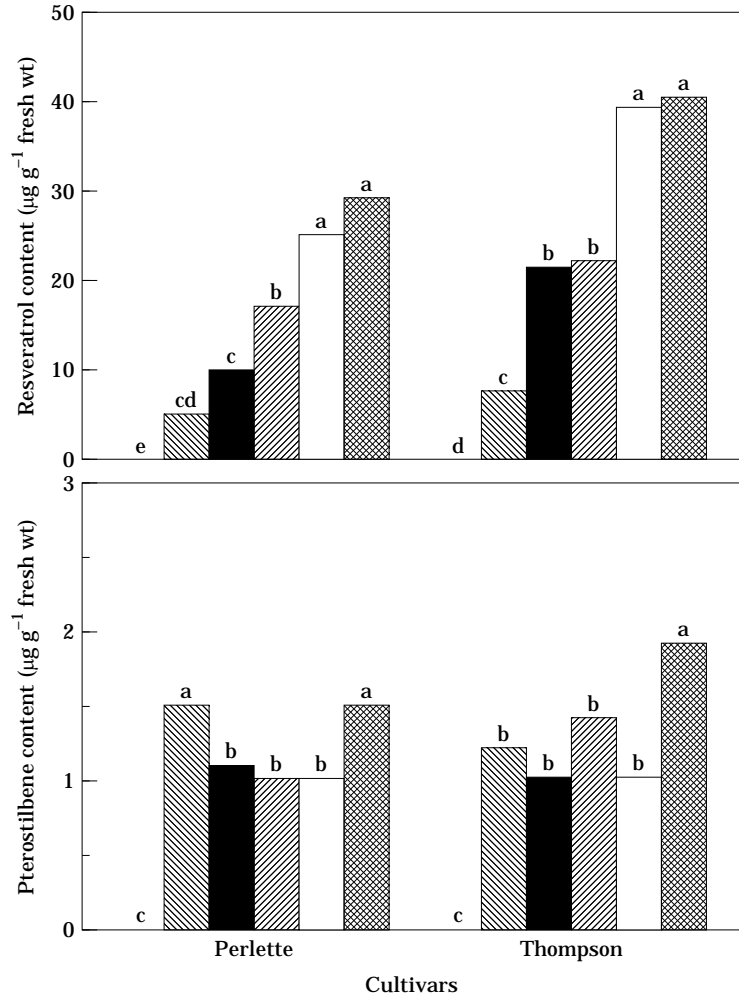


FIG. 5. Effects of exposure to ozone, uv irradiation and fungal inoculations on the elicitation of resveratrol and pterostilbene in grape berries of cvs. Perlette and Thompson Seedless. Control (□); *Botrytis* inoculation (▨), *Rhizopus* inoculation (■), ozone (▧), uv, 5 min (□) and uv, 10 min (▩). Different letters indicate significant differences for each compound in each cultivar according to Duncan's multiple range test ($P \leq 0.05$).

A comparison of SO₂ and ozone for the control of berry decay during and following cold storage
 Experiments were performed to compare a pre-storage exposure to ozone with a continuous SO₂ supply during storage from a two stage generator [13]. Decay severity on untreated fruit stored for 5 weeks at 0 °C and 3 days at 20 °C was high, but did not increase with longer storage periods (Table 1). It was caused mainly by two fungi—*B. cinerea* and *R. stolonifer*, the former during storage and the latter during shelf-life. Results showed that both ozone and SO₂ treatments decreased decay severity significantly. Although the differences between the two treatments were not significant, the extent of decay in the SO₂ treatment was always less than in the ozone-treated fruit

and the overall appearance of SO₂ treated fruit was generally superior. After 9 weeks of storage, the decay severity and fruit quality were equivalent in both lots of treated fruit, which were significantly superior to untreated controls.

Comparison between effectiveness of ozone exposure, uv-c irradiation and fungal inoculations in phytoalexin elicitation in grape berries

The relative effectiveness of exposure to ozone, uv irradiation and fungal infection in eliciting resveratrol and pterostilbene production was compared in two grape cultivars (Fig. 5). Resveratrol was elicited in greater amounts than pterostilbene by all treatments. Inoculations with *Rhizopus* elicited more resveratrol than *Botrytis*, and both fungi elicited less phytoalexin than exposure to uv. No differences were found between uv irradiation for 5 or 10 min in elicitation of resveratrol. Levels of resveratrol elicited by ozone were between those elicited by uv irradiation and fungal inoculations. Pterostilbene content was just above the detection level and differences between treatments were difficult to discern. However, uv irradiation for 10 min was more effective than other treatments. No phytoalexins were detected in untreated controls.

DISCUSSION

The effectiveness of a single, short post-harvest exposure of table grapes to ozone for the control of storage cluster decay (Table 1), is apparently due to both its direct fungicidal effect on the decay-causing organisms (Fig. 2) and its ability to elicit stilbene phytoalexins in the grape berries (Fig. 4). A 10-min exposure to ozone was at least as effective as immersing the fruit in ethanol for reducing the microbial population inhabiting harvested berries. The bactericidal effect is probably irrelevant, as no known decay is caused by bacteria. With regard to yeasts, they are often found to inhabit decayed fruit, but have not been shown to be the initial causal organisms of decay. Their reduction on the fruit surface may even be detrimental, since many of the yeast species found on plant surfaces are beneficial for biological control of decay-causing fungi [9, 10].

Total sterilization of the fruit surface after harvest can presumably provide complete protection of the fruit from decay development during prolonged storage, as evidenced by the effect of immersion in 70% ethanol (Figs. 2 and 3). Even when ethanol alone did not completely eliminate all micro-organisms from the fruit surface, decay control was almost complete. This is somewhat surprising, as one would expect decay to develop in storage either from re-infection from the spore-laden atmosphere or from latent infections by *B. cinerea* [15]. In view of the fact that ethanol did not elicit stilbene production, but even inhibited phytoalexin elicitation by ozone (Fig. 4), we have to assume that it either induced a different defense mechanism or that surface sterilization before storage protected the fruit from subsequent decay development. If the latter is true, it is conceivable that the effect of ozone in controlling storage decay was also due to sterilization of the fruit surface prior to storage. Ethanol inhibition of phytoalexin elicitation by ozone is of interest and may be an indication of the elicitation pathway. Recent findings have been reported on the existence of a resveratrol glucoside in grape berries [41] and in wine [17]. In the latter report, addition of β -glucosidase increased resveratrol levels in the wine. This enzyme has been found in grapes and it was shown that its activity can be reduced in the presence of ethanol [1]. We therefore speculate

that the reduced resveratrol levels in ethanol-treated fruit subjected to ozone exposure, might be an indication that the glucoside precedes resveratrol elicitation by ozone.

A good correlation can be observed between the size of the fungal population remaining on the fruit following ozone treatment and the extent of storage decay on non-inoculated fruit (Figs. 2 and 3). However, the control of decay development in fruit inoculated with *R. stolonifer* after exposure to ozone (Fig. 3), points to the elicitation of a defense mechanism. In this instance, decay control can be attributed to ozone elicitation of resveratrol and pterostilbene, both of which are correlated with the relative resistance of grapes to fungal decay [16, 30, 31, 35]. The enhancement of total stilbene production, when inoculated berries were exposed to ozone after inoculation (Fig. 4), correlates well with the extent of fungal decay control achieved following up to 30 min exposure (Fig. 3).

Ozone induction of plant defense has been attributed to different systems in different plants, and has been recently thoroughly reviewed by Kangasjärvi *et al.* [18]. Generally, they appear to be closely related to the pathogen induced defense responses. These systems include the induction of PR proteins, of ethylene and polyamine synthesis, of enzymes capable of detoxifying the active oxygen species it itself produces [14, 19], and of the phenylpropanoid pathway. So far we have studied only the last of these possibilities and the involvement of the others cannot be ruled out. In tobacco, for example there is evidence for the existence of these systems [37], and in parsley, ozone treatment resulted in time-dependent induction of genes for PR proteins, the phenylpropanoid pathway and detoxification mechanisms [11]. The question as to what is the signal for stilbene synthesis is open to speculation. Deighton *et al.* [7] found no evidence to support the idea that active oxygen species are responsible for elicitation of phytoalexins during the interaction of *Glycine max* cotyledons with *Erwinia carotovora*. Other potential signal transducers which have been proposed, such as ethylene [26], jasmonic acid [18] and salicylic acid [43] are currently being studied in a model system of ozone-exposed grape cell suspensions.

The response to ozone is very similar to stilbene elicitation in grapes by uv-c irradiation, with regard to the relative levels of resveratrol and pterostilbene (Fig. 5), and the duration of exposure generally required for maximum effect (10 min). This was to be expected, since there are a number of similarities in the response of terrestrial vegetation to both factors, but the interactions between them often make it difficult to unequivocally define the individual effect of each [33]. However, the somewhat greater efficiency of exposure to uv-c in eliciting resveratrol appears to preclude the possibility that it is acting *via* the small amounts of ozone which the uv lamp may produce [40].

The results of a trial conducted with several boxes of fruit, cold-stored for up to 9 weeks, indicate that a short-term postharvest exposure of grapes to ozone might be a commercially satisfactory alternative to SO₂. Not only was decay controlled by ozone as effectively as by SO₂, but the quality and freshness of the treated fruit were superior to those of the untreated control fruit. However, the SO₂ treated fruit was generally of better appearance due to the bleaching of the stems and pedicels, which imparts a fresh look even when these parts are actually relatively dry.

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