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Effect of a continuous low ozone exposure (50 nL L^{-1}) on decay and quality of stored carrots

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ABSTRACT

Fresh carrots were treated with or without a continuous atmosphere of 50 ± 10 nL L^{-1} ozone during storage for up to 6 months at 0.5 °C and >95% relative humidity to determine the effect on decay caused by *Sclerotinia sclerotiorum* and *Botrytis cinerea* and quality attributes. Lesions on carrots inoculated with *S. sclerotiorum* at the beginning of the storage period were reduced in length but the subsequent rate of lesion expansion over time was similar for both treatments. Lesion size and rate of expansion on carrots inoculated with *B. cinerea* were reduced by ozone. Aerial mycelium of both pathogens was markedly reduced in the ozone treatment, but sporulation of *B. cinerea* was stimulated, characterized by dense mats of short conidiophores on the lesions. Susceptibility to *S. sclerotiorum* increased with storage time while susceptibility to *B. cinerea* peaked at 4 months and then decreased possibly related to carrot moisture loss. The incidence of carrots harboring visible saprophytic mold on the crown was substantially reduced in the ozone treatment. Ozone-induced injury, appearing as blotches of brownish discolored periderm, was slight, but increased with time whereas carrots in the control treatment did not become discolored. Ozone treatment had no effect on fresh weight loss, sprouting of carrot crowns, nor on concentrations of glucose, fructose, sucrose or galactose. Levels of isocoumarin (3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin) were slightly elevated and averaged 17.8 mg kg^{-1} compared with 12.1 mg kg^{-1} in the controls and may have been associated with reduced lesion growth by *B. cinerea*. The ozone treatment may be useful for reducing nesting caused by *S. sclerotiorum* and *B. cinerea* in carrots destined for the processing market where any ozone-induced discoloration would be removed during peeling.

1. Introduction

Storage rots of carrot (*Daucus carota* L.) caused by *Sclerotinia sclerotiorum* (Lib.) de Bary (watery soft rot) and *Botyrtis cinerea* Pers.:Fr. (grey mold) are significant problems in many carrot producing areas of the world (Rader, 1952; Lockhart and Delbridge, 1972; Goodliffe and Heale, 1975; Kora et al., 2003). Both pathogens have their origin in the field and the storage phase is difficult to control. In the field, both pathogens colonize the foliage and grow down the petiole to the crown where they remain as incipient infections and, with time in storage, begin to colonize and decay root tissues (Goodliffe and Heale, 1975; Finlayson et al., 1989; Kora et al., 2005). Both pathogens may cause pockets of diseased carrots or a 'nesting effect' in bulk storage where mycelium spreads among carrots in close contact resulting in substantial losses. Although field-applied fungicides may be effective in reducing the incidence of infected roots going into storage (Tahvonen, 1985), there are no fungicides available to specifically control the postharvest phase of these pathogens. As an alternative, we have been exploring the use of gaseous ozone for postharvest disease control.

Ozone, the tri-atomic form of oxygen, is a strong antimicrobial agent and is now approved for use on food by the FDA (Federal Register, 2001). Ozone has been reported to reduce decay of some fruits and vegetables, but results have been variable (Forney, 2003). In early work on carrots, Liew and Prange (1994) showed that ozone could reduce decay caused by *B. cinerea* and *S. sclerotiorum*, but high concentrations cause extensive physiological injury to the roots. They suggested that a concentration of 15 μL L^{-1} for an 8-h daily exposure might be suitable to provide disease protection and avoid toxicity to the carrots. We have tested exposures of 1000 and 300 nL L^{-1} for 1, 2 or 4 d prior to storage (Song et al., 2003; Forney et al., 2007). Resistance to *B. cinerea* colonization is induced by treatment for 2 and 4 d with 1000 nL L^{-1}, but not to *S. sclerotiorum*. However, elevated levels of isocoumarin 6-methoxymellein (3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin; 6-MM) also occurs with these treatments. While 6-MM is associated with resistance to *B. cinerea* (Goodliffe and Heale, 1978; Harding and Heale, 1980; Hoffman and Heale,

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1989; Mercier et al., 1993), it is also known to be the cause of bitterness in carrots (Sondheimer, 1957). Exposure to ozone for 2 or 4 d at 1000 and 300 nL L^{-1} for 4 d also causes noticeable and slight discoloration, respectively. Other physiological perturbations also occur with these treatment regimes indicating that these ozone concentrations and exposure times are still too high to avoid injury and yet control disease (Forney et al., 2007). Treatment with 1-methylcyclopropene (1-MCP), an ethylene receptor blocking agent (Blankenship and Sisler, 1993) tends to reduce the adverse physiological consequences of the ozone treatments, but this effect is not adequate to maintain good carrot quality (Forney et al., 2007).

Therefore, the objectives of the present study were to test the effects of a continuous exposure to ozone, at a concentration lower than previously tested, on the colonization of carrot tissue by *S. sclerotiorum* and *B. cinerea* during cold storage and to examine physiological responses of the carrot tissue.

2. Materials and methods

2.1. Storage conditions

In November 2001, four carrot cultivars 'Vita Treat', 'Triple Play', 'Health Master', and 'Pot o' Gold' and in November 2003, four cultivars 'Vita Treat', 'Arrowhead', 'Dyna Peel' and 'Tempo' were harvested from a cultivar trial not associated with this study. The carrots were obtained from two replicates in each of two commercial field plots located near Berwick and Canning, Nova Scotia.

The carrots were held in two treatment chambers (6.2 m^3) constructed of wooden frames lined with 0.15 mm polyethylene film. The chambers were situated in a walk-in controlled environment chamber (Model GR36, Enconaire Systems Ltd., Winnipeg, MN, Canada) operating at 0.5 ± 0.2 °C. A small fan within each chamber ensured mixing of air and a uniform temperature.

One chamber was designated as the control chamber and in the other an ozone generator (Model SF 300, Aqua-air, Simpson Environmental Corp., ON, Canada) was installed. A continuous ozone concentration of 50 ± 10 nL L^{-1} was established and monitored with an ozone analyzer (Model 1180, Dasibi Environmental Corp. Galendale, CA, USA) operating in conjunction with a datalogger (Model 21X, Campbell Scientific Canada Corp., Edmonton, AB, Canada). The ozone concentration was maintained by using an electronic relay controlled by programmed set points in the datalogger to turn the generator on and off. Temperature was monitored with thermocouples at various positions throughout each chamber and relative humidity (RH) was monitored with a probe (HMP45C, Campbell Scientific Canada Corp., Edmonton, AB, Canada) positioned in the center of each chamber. The carrots were placed in slatted wooden flats that were first soaked in water prior to storage and the sides and floor of the chambers were wetted at biweekly intervals to maintain >95% RH during storage.

2.2. Inoculated carrots

At harvest, the petioles of carrots to be inoculated with *S. sclerotiorum* were cut 1 cm from the crown while all other carrots were topped at the crown. At the outset of the experiment, five carrots per cultivar and field replicate were inoculated with *S. sclerotiorum*. An agar plug (6 mm) obtained from a 2-d old potato dextrose agar (PDA) culture of the pathogen was placed at the base of the petioles of each carrot. The carrots were then placed in plastic bags with a moist paper towel and incubated at 20 °C for 24 h to initiate infection. Following incubation, the agar plugs were removed and the carrots were laid individually in the flats situated in the control and ozone chambers. At monthly intervals for 6 months, the distance between the crown and the leading edge of visible mycelium and/or decayed tissue was measured.

Also, after 3 months of storage, 10 healthy carrots of each cultivar and field replicate from the control chamber were inoculated with *B. cinerea*. A 6-mm diameter agar plug from a 4-d old PDA culture was placed into a 5-mm deep cavity created with a 6-mm diameter cork borer near the center of each carrot. After 48 h of incubation at 20 °C, the agar plugs were removed and five carrots were returned to each of the control or ozone chambers. Lesion lengths along the carrot axis were measured at monthly intervals for the next 3 months. These carrots were inoculated after 3 months of storage because susceptibility to *B. cinerea* is low upon initial storage and increases with time (Goodliffe and Heale, 1977; Forney et al., 2007).

Five noninoculated carrots of each cultivar and field replicate from the control and ozone chambers were also challenge inoculated at monthly intervals to determine if the ozone treatment induced resistance to infection. Carrots were inoculated with *S. sclerotiorum* or *B. cinerea* as above and incubated at 4 °C for 8 weeks, after which lesion lengths were measured. For this experiment, the agar inoculum plugs were not removed.

In all experiments, tissues radiating outward about 1–2 mm from the agar plugs of *S. sclerotiorum* and *B. cinerea* appeared water soaked indicating that both pathogens were established at the inoculation sites before the carrots were transferred to the treatment chambers.

2.3. Quality analysis

Nylon mesh onion bags containing 30 noninoculated carrots were also stored in the flats and removed from the chambers at monthly intervals for 6 months. At each removal, carrots from each bag were evaluated against a number of quality parameters. The incidence of carrots with decay, saprophytic mold growth at the crown and the incidence of shoot growth was assessed. Fresh weight loss in relation to initial weight of each bag was also measured. Ozone-induced injury was rated on a scale of 0–2 where 0 = none; 1 = slight; and 2 = moderate for each carrot.

Ten random healthy carrots from each bag were then peeled and this tissue was frozen in liquid N_2 and stored at −40 °C prior to extraction and analysis of sugars and 6-MM. The method of Seljåsen et al. (2001) was followed with a few modifications. Sugars and 6-MM were extracted by placing a 5-g frozen sample into a glass tube, to which 20 mL of boiling water was added. The sample was held in a boiling water bath for 10 min. The extract was then filtered (Q8 filter paper, Fisher Scientific, Pittsburgh) and another 5 mL of boiling water was used to wash the sample. Total extract solutions were weighed and loaded onto a C_{18} silica gel column (Sep-Pak cartridge, 0.85 mL, Waters, Milford, MA, USA) that had been conditioned with 4 mL of methanol followed by 10 mL of water. The first 14 mL of the extracted solution was collected in a 15 mL polypropylene tube (Corning Inc., Corning, NY, USA) and stored at −40 °C for sugar analysis. The column was then washed with 5 mL of water and air dried. The 6-MM was eluted with 1.9 mL of methanol and collected into a 4-mL brown glass vial. The net weight of the eluate was recorded and held at −20 °C.

For sugar analysis, a 0.5 mL sample of the water eluate from the C_{18} column was filtered through a 0.22-cm filter (Chromatographic Specialties Inc., Brockville, ON) and analyzed for glucose, fructose, sucrose, and galactose. Samples were analyzed on a carbohydrate column (Rezex RPM Monosaccharide, 300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA) at 85 °C using a refractive index detector (model 156, Beckman, Fullerton, CA or 2414 RI, Waters Limited, Mississauga, ON, Canada) at 40 °C. The mobile phase was

18.2 MΩ cm water (MilliQ-uRF, Millipore Ltd., Nepean, ON, Canada) at 85 °C and a flow rate of 10 μL s^{-1}. Samples were quantified using external standards (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada).

For 6-MM analysis, 0.5 mL of the methanol eluates were filtered through 0.22-μm filters (Chromatographic Specialties Inc., Brockville, ON, Canada) prior to analysis. Samples were analyzed on an HPLC (System Gold, Beckman, Fullerton, CA, USA), equipped with a silica column (Supelcosil, LC-18, 300 mm × 4 mm and 5 μm, Supelco, Bellefonte, PA or Synergi Hydro-RP, C-18, 250 mm × 4.6 mm and 4 μm, Phenomenex, Torrance, CA, USA) at 30 °C and an UV-detector at a wavelength of 267 nm with a 4 nm bandwidth. Methanol/water (60:40 v/v) was used as the mobile phase at a flow rate of 16.7 μL s^{-1}. A sample of 6-MM (94% purity) received from Dr. S. Talcott (University of Florida), was used as an external standard.

2.4. Statistical analysis

The treatment and control chambers were interchanged in each year of the study. Carrot flats were stacked in groups of four within each chamber, and cultivars and field replicates were appropriately randomized using a Latin Square design. Lesion lengths and the injury rating were transformed to the square root scale, incidence of carrots with saprophytic mold and shoots were transformed to angles, and 6-MM levels were transformed to the $\log_{10}$ scale before analysis using the ANOVA directive in Genstat 7.1 (Payne, 2003). A repeated measures analysis was used to quantify treatment effects on lesion size of carrots inoculated at the beginning of the experiment with the same carrots being repeatedly measured over the course of the storage period. When appropriate, response variates were regressed over storage time. Because the ANOVA did not indicate cultivar by treatment interactions, data were averaged over cultivars. Cropping years were treated as replicates. The probability level for significance testing was set at $P \leq 0.05$. Standard errors of the mean (SEM) for regression parameters are reported in the figures and the backtransformed scale is presented on the right vertical axis of each figure.

3. Results

3.1. Inoculated carrots

One month after carrots were inoculated with *S. sclerotiorum*, lesion lengths were reduced by continuous exposure to ozone compared with the nontreated controls (Fig. 1). Thereafter, lesions remained smaller on the treated carrots, but the rate of lesion expansion was similar for both treatments. Little aerial mycelium was observed on the inoculated petioles of the ozone-treated carrots compared with the controls after the first month and, thereafter, mycelium was also reduced on root tissues even though lesions advanced. However, the number and size of sclerotia that developed on the carrots was generally similar in both treatments by the end of the storage period.

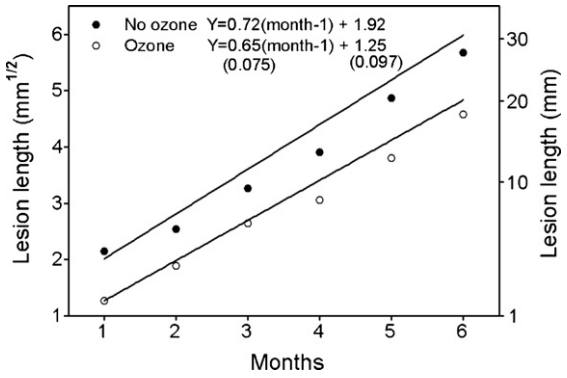

Fig. 1. Lesion length on carrots inoculated with *S. sclerotiorum* and stored for 6 months at 0.5 °C in a continuous atmosphere of 50 ± 10 nL L^{-1} gaseous ozone or in an ozone-free atmosphere. Standard errors of the regression coefficients are presented in parentheses below each term. Data were analyzed using repeated measures and so only a single SEM is presented.

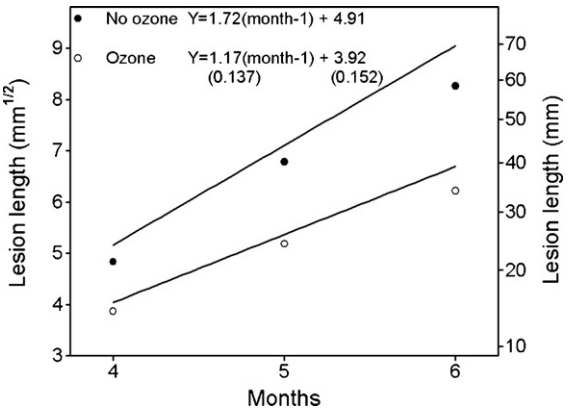

Fig. 2. Lesion length on carrots inoculated with *B. cinerea* after 3 months of storage at 0.5 °C and subsequently stored at 0.5 °C in a continuous atmosphere of 50 ± 10 nL L^{-1} gaseous ozone or in an ozone-free atmosphere. Standard errors of the regression coefficients are presented in parentheses below each term. Data were analyzed using repeated measures and so only a single SEM is presented.

Lesion lengths on carrots stored for 3 months and then inoculated with *B. cinerea* were also reduced by the ozone treatment after the first month of ozone exposure, but unlike with *S. sclerotiorum*, the rate of lesion expansion over time was lower compared with the controls resulting in substantially smaller lesions at the end of the storage period (Fig. 2). Aerial mycelium on lesions was also notably shorter in the ozone treatment. No spore production was observed in either treatment during the first 2 months of storage, but after 3 months, spore production was noticeably greater in the ozone treatment. Dense mats of short conidiophores occurred on lesions of treated carrots compared with scattered conidiophores occurring in the fluffy aerial mycelium of the control carrots.

The ozone treatment did not appear to induce resistance to *S. sclerotiorum* and *B. cinerea* as measured by lesion length on carrots that were challenge inoculated after monthly intervals of storage. Susceptibility to *S. sclerotiorum* was similar in both the control and ozone-treated carrots and continued to increase with storage time (Fig. 3a). Susceptibility to *B. cinerea* was also similar in both treatments, but increased to a maximum after 4 months of storage and then decreased (Fig. 3b).

No decay was observed on any of the noninoculated carrots, but saprophytic molds established quickly after the start of the storage period initially appearing as sparse mycelial growth on the crowns of carrots. With time, mycelial density increased, but did not cause decay. The incidence of carrots with saprophytic mold after the first month of storage was substantially reduced by the ozone treatment, and thereafter, the rate of increase of affected carrots over time was also lower compared with the controls (Fig. 4).

3.2. Quality analysis

The ozone treatment caused some injury which appeared as scattered blotches of slightly brown discolored periderm. The injury ratings of the ozone-treated carrots, while low, increased over the storage period (Fig. 5).

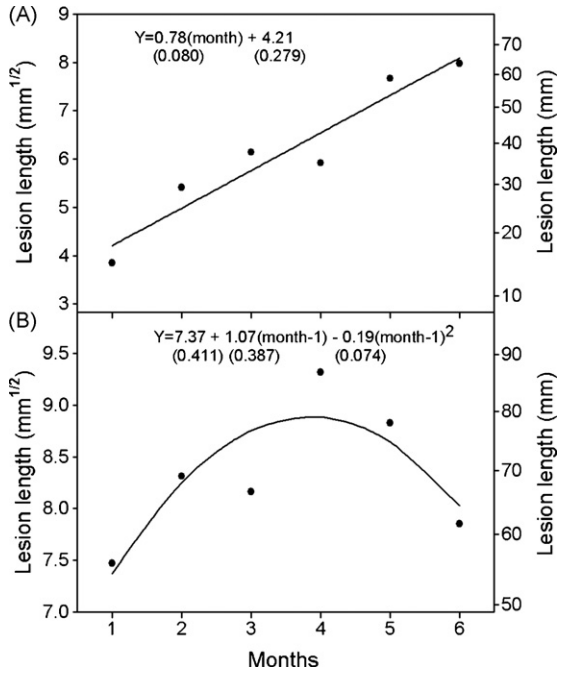

Fig. 3. Lesion length on carrots challenge inoculated with (A) *S. sclerotiorum* and (B) *B. cinerea* at monthly intervals after storage at 0.5 °C in a continuous atmosphere of 50 ± 10 nL L^{-1} gaseous ozone or in an ozone-free atmosphere. Following inoculation, carrots were incubated at 4 °C for 8 weeks after which lesion lengths were measured. Data are averaged over the ozone and control treatments since the ANOVA indicated no treatment effects. Standard errors of the regression coefficients are presented in parentheses below each term.

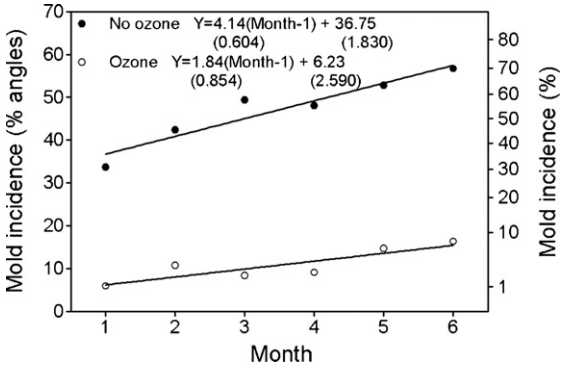

Fig. 4. Incidence of saprophytic mold on crowns of carrots stored at 0.5 °C for up to 6 months in a continuous atmosphere of 50 ± 10 nL L^{-1} gaseous ozone or in an ozone-free atmosphere. Standard errors of the regression coefficients are presented in parentheses below each term.

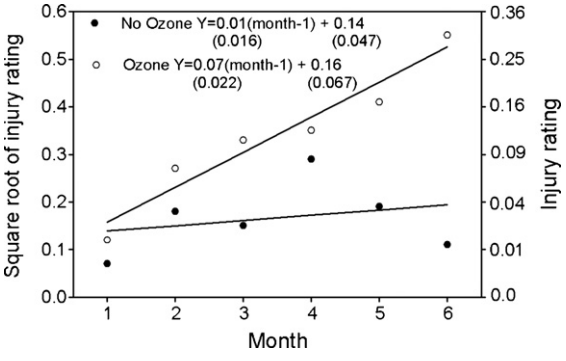

Fig. 5. Injury rating (0–2) of carrots stored at 0.5 °C for up to 6 months in a continuous atmosphere of 50 ± 10 nL L^{-1} gaseous ozone or in an ozone-free atmosphere. Standard errors of the regression coefficients are presented in parentheses below each term.

Ozone had no effect on several of the other quality parameters investigated and so the data were averaged over treatments. Fresh weight loss increased linearly to 10.2% after 6 months of storage. Sprouting on carrots remained low (≤0.8%) until after 5 and 6 months of storage when the incidence increased markedly to 18.9% and 24.7%, respectively. Glucose and fructose concentrations increased slightly and linearly from respective mean concentrations of 7.3 and 6.3 g kg^{-1} after 1 month of storage to 10.4 and 8.6 g kg^{-1} after 6 months. Sucrose concentration remained constant at 23.3 g kg^{-1} while galactose increased linearly from 0.9 to 1.4 g kg^{-1} after 1 and 6 months of storage, respectively.

Concentrations of 6-MM were slightly, but significantly elevated in the ozone-treated carrots and averaged 17.8 mg kg^{-1} compared with 12.1 mg kg^{-1} in the controls, but they remained constant throughout the storage period.

4. Discussion

The ozone treatment significantly reduced the size of lesions incited by both *S. sclerotiorum* and *B. cinerea* during the first month following inoculation (Figs. 1 and 2). In addition to the water soaking which occurred around the agar inoculum plugs indicating that infection was established, mycelium was also observed growing on the carrot tissues around the inoculation point. It is possible that the ozone killed or restricted this surface mycelium which resulted in smaller lesions. However, in the case of *S. sclerotiorum*, the rate of lesion expansion over storage time was similar for both the ozone and control treatments indicating that ozone had no effect on subsequent disease development; the pathogen was able to colonize unimpeded in the protected cell layers beneath the epidermis of petioles and subsequently beneath the periderm of the root. The inability of either gaseous ozone or ozone dissolved in water to penetrate tissues and control established infections has been observed previously in numerous studies (Spalding, 1968; Spotts and Cervantes, 1992; Smilanick et al., 1999; Hildebrand et al., 2001; Palou et al., 2001, 2002; Nicoué et al., 2004).

In the case of *B. cinerea*, the rate of lesion expansion was reduced and possibly related to the induction of 6-MM. We have previously shown that ozone can illicit 6-MM accumulation (Song et al., 2003) which is known to inhibit *B. cinerea* (Goodliffe and Heale, 1978; Harding and Heale, 1980; Hoffman and Heale, 1989; Mercier et al., 1993). In the present study, the ozone-induced increase that occurred, while not high (17 mg kg^{-1}), was significant and may have contributed to the reduced rate of lesion expansion. Mercier et al. (1993) reported that a concentration of just 30 mg kg^{-1} inhibited *B. cinerea*. However, in the experiment in which carrots were challenge inoculated at monthly intervals after exposure to ozone, an induction of resistance was not evident (Fig. 3), but these carrots were incubated at a higher temperature of 4 °C which may have allowed the pathogen to overcome any slight level of resistance induced by the ozone.

In this same experiment, the level of susceptibility to *B. cinerea* as measured by lesion size, increased to a maximum after 4 months in storage and then declined whereas the susceptibility to *S. sclerotiorum* continued to increase with time (Fig. 3). Goodliffe and Heale (1977) showed that susceptibility to *B. cinerea* increases markedly with an age-associated fresh weight loss of >5%, but they examined this relationship for only a 3-month storage period. In our study, the carrots had lost 3.5, 4.8, 6.1, 7.7, 8.8 and 10.2% of their fresh weight after each of the 6 months in storage. It is possible that *B. cinerea* is less able to colonize carrot tissues compared with *S. sclerotiorum* after a threshold of reduced water content occurs. Interestingly, Goodliffe and Heale (1977) also reported that lesions of *B. cinerea* were only about 10–11 and 15–16 mm in length along the roots from the point of inoculation after 1 and 3 months of

storage, respectively, compared with lesion diameters of 54.3 mm (7.37 $mm^{1/2}$) and 76.6 mm (8.75 $mm^{1/2}$) after the same periods in our study (Fig. 3B). The reason for this large difference in lesion size is not obvious since both studies employed a similar inoculation technique and incubation temperature and period. However, it may be possible that the cultivars used in our study were more susceptible than those used by Goodliffe and Heale (1977) and/or our isolate of *B. cinerea* was more virulent.

Previous studies have shown that ozone at high concentrations causes pitting and bleaching of tissues (Liew and Prange, 1994) and at lower concentrations, injury is less severe and appears as blotches of brown discolored periderm (Forney et al., 2007). The fact that ozone at only 50 $nL\,L^{-1}$ in the present study still caused some discoloration indicates that carrot is highly susceptible to the oxidative action of ozone and that perhaps no safe level of exposure may be achievable. However, the ozone treatment did not result in other physiological perturbations as occurred in the earlier studies in which higher concentrations were used (Liew and Prange, 1994; Forney et al., 2007).

While the low ozone concentration did not completely control decay, disease severity was significantly reduced. The ozone-induced discoloration that occurred, though slight, would likely be noticed by consumers and would not be acceptable in a whole carrot pack. However, a low ozone concentration might nevertheless be useful commercially to reduce nesting caused by *S. sclerotiorum* and *B. cinerea* in carrots stored and destined for the processing market. We have previously shown that ozone at higher concentrations substantially reduces aerial mycelium of *S. sclerotiorum* and *B. cinerea* (Hildebrand et al., 2001) and this effect also occurred at the low ozone concentration in the present study. In addition, the incidence of roots harboring saprophytic molds was also substantially reduced by the ozone treatment (Fig. 4) indicating that carrot-to-carrot mycelial spread of the pathogens would be reduced. Interestingly, the ozone treatment stimulated spore production by *B. cinerea* in lesions which contrasts with previous work where higher concentrations of ozone have been shown to reduce spore production (Hildebrand et al., 2001; Palou et al., 2002). Although a stimulating effect was observed, spores of this pathogen do not infect the carrot periderm unless it is wounded (Goodliffe and Heale, 1977). Harvest-induced wounds heal rapidly and become resistant to infection when carrots are held at 15–25 °C and high humidity for 2 d (Sharman and Heale, 1977; Harding and Heale, 1981; Lewis et al., 1981). Under cold storage conditions, harvest-induced wounds would heal more slowly but likely would be complete by the time spores from lesions would develop and be dispersed. Spores were not produced on infected tissues until the third month after inoculation in the present study. Thus, an ozone-induced increase in airborne spore numbers might not be a problem, though this requires further investigation. In addition, any ozone-induced discoloration of the periderm would be removed during peeling and the slightly elevated levels of 6-MM would not be detected by the human palate as levels in this study reached only 17 $mg\,kg^{-1}$ which is well below the perception threshold of 94 $mg\,kg^{-1}$ (Talcott and Howard, 1999). Moreover, most of the 6-MM that accumulates in carrot occurs in the peel (Lafuente et al., 1996).

To be effective commercially, ozone must be able to penetrate into the center of carrot bins or piles to reduce nesting caused by *S. sclerotiorum* and *B. cinerea*. With citrus, Harding (1968) showed that ozone at a concentration of 1000 $nL\,L^{-1}$ could penetrate cardboard cartons with large but not small openings and Palou et al. (2001) reported good penetration of ozone at a lower concentration of 300 $nL\,L^{-1}$ in large plastic field bins with large vents. Because ozone is rapidly depleted on organic surfaces (Forney, 2003) it is not known whether a much lower ozone concentration as currently studied (50 $nL\,L^{-1}$) could be achieved and maintained in the center of carrot bins or piles commonly used in commercial storages. Thus, further experiments would be needed to develop and test methods of ozone delivery under such conditions.

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