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Aqueous ozone can extend vase-life in cut rose

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SUMMARY
In order to quantify the shelf-life response of cut roses when stored in aqueous ozone solutions, cut 'Pascha' roses were stored in either de-ionised water or aqueous ozone solutions containing an initial dissolved ozone residual of 5.5 mg l^{-1}. The results showed that storing cut roses in aqueous ozone solutions (5.5 mg l^{-1}; renewed daily) can extend vase-life approx. three-fold, from 5 d to 13 d, with a corresponding improvement in their aesthetic appearance throughout the vase-life of the cut rose stem. Results suggest that vase-life improvements are achieved through a reduction in bacterial populations present in the storage solution. Bacteria accumulate on the cut surface of the stems, thereby reducing their water uptake capacity. Microbial accumulation was reduced by 1.15 $\log_{10}$ CFU g^{-1} FW when stems were stored in holding solutions containing 5.5 mg l^{-1} dissolved ozone, with a corresponding increase in water uptake. Roses stored in ozonated water exhibited higher numbers of functional xylem vessels, water uptake, relative water content, relative fresh weight, acid fuchsin uptake rate, leaf stomatal conductance, and net CO_2 assimilation rate, compared to those stored in de-ionised water. The results suggest that ozone can extend cut rose vase-life.

After harvest, cut rose stems are known to develop blockages in their xylem vessels, thereby impeding water uptake and negatively affecting vase-life (Robinson *et al.,* 2007). Generally, it is thought that this development of vascular occlusions is caused by the presence of high numbers of bacteria in the holding solution (Robinson *et al.,* 2007). Thus, measurement of water uptake capacity is a key indicator parameter when evaluating the efficacy of any cut flower preservative.

Ozone is a strong oxidising molecule which is used as an anti-microbial agent in numerous applications, including the treatment of sewage and drinking water, disinfection of biological safety cabinets, and food preservation. Upon decomposition, in water, ozone produces diatomic oxygen and a number of free radicals such as superoxide, hydroperoxyl, and hydroxyl radicals (Staehelin *et al.,* 1984). These free radicals react rapidly with organic and inorganic compounds in the solution. In many applications, ozone is favoured over other oxidising agents (e.g., chlorine) due to its low probability of forming toxic by-products and its relatively short half-life.

This study was designed to evaluate the efficacy of aqueous ozone in extending the vase-life of cut rose. It was hypothesised that aqueous ozone was capable of reducing the concentration of bacteria in cut rose holding solutions; bacteria which are known to cause xylem occlusions. In addition, it was hypothesised that, by reducing the bacterial population, the vase-life of cut roses would be prolonged as such conditions would provide an optimal environment for maintaining key physiological processes in cut rose. The objectives of this study were: (i) to determine if aqueous ozone could be effective in reducing bacterial numbers in the holding solution of cut roses; and (ii) to quantify selected physiological responses (e.g., vase-life) of cut roses to aqueous ozone.

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MATERIALS AND METHODS

Plant material and ozone treatment

Flowering rose stems (*Rosa hybrida* L. 'Pascha') were harvested and supplied by Thiessen Greenhouse Flowers Ltd. (Leamington, Ontario, Canada; 42° 2'N, 82° 34'W). Stems were transported (total distance = 300 km) on ice to the laboratory at the University of Guelph (43° 33'N, 80°15'W) and immediately recut to a standard length of 50 cm, under de-ionised water, to remove any air that may have entered the stems during transport. Stems were stored at 4ºC in de-ionised water until required.

Leaves were removed, up to the uppermost five leaflets. Each stem was then placed in a glass cylinder containing 200 ml of de-ionised (n = 45 stems) or ozonated (n = 45 stems,) water as holding solution in a climate-controlled chamber. Ozonated water contained 0.5, 1.0, 2.2, 4.0, or 5.5 mg l^{-1} ozone, respectively. The holding solution in each glass cylinder was renewed daily. The chamber was controlled to 20ºC and 60% relative humidity, and was illuminated from 0600 – 2200 h at a photosynthetically active radiation (PAR) level of 550 µmol m^{-2} s^{-1} *via* high pressure sodium lamps. The CO_2 concentration was held at 400 µmol mol^{-1}. To ensure a similar and relatively high number of bacteria in the holding solutions, 300 µl of a leaf 'slurry', prepared by homogenising 10 g rose leaves in 100 ml de-ionised water for 3 min, was also added to each cylinder, each day.

Ozone solutions were generated with a Central Purification System (CPS; Purification Research Technologies Inc., Guelph, Ontario, Canada). The system generated ozone *via* a corona discharge and continually transferred the gas to a mixing loop for on-demand use. Ozone concentrations were monitored using a low concentration ozone analyser (W1; IN USA Ltd., Needham, MA, USA), which was checked for accuracy using the indigo colorimetric method (Bader and Hoigne, 1981; Gordon and Bubnis, 2002).

Vase-life

Roses were considered to have reached the end of their vase-life when any of the following symptoms developed: petal or leaf wilting (flaccid petals or leaves), petal or leaf abscission, development of bent neck (90º bend at the peduncle), or petal discolouration. Roses were evaluated daily for the onset of any of the above symptoms.

Bacterial counts

Bacterial growth curves ($\log_{10}$ CFU ml^{-1}) in both de-ionised and ozonated holding solutions containing a cut rose, were determined using the standard plate-count method, with samples being drawn at 0, 3, 6, 12, and 24 h after each holding solution change. Bacteria were plated on Trypticase Soy Agar (TSA; Difco Media; Fisher Scientific, Whitby, Ontario, Canada) in 10 cm pre-sterilised Petri plates (Fisher Scientific) and incubated at 30ºC for 48 h. TSA was chosen as it is a general purpose medium for collecting and counting a wide variety of microorganisms.

On day-5, the numbers of bacteria in the basal 1 mm of each stem (i.e., the cut surface) were counted for both treatments, as described by van Doorn and de Witte (1997) with modifications. Briefly, the bottom 1 mm of each stem was removed using a sterile razor-blade. The excised stem fragment was placed in 10 ml sterile 0.85% (w/v) NaCl solution and shaken at 2,500 rpm for 3 min. Dilutions were plated on TSA and incubated at 30ºC for 48 h.

Water uptake

Water uptake in six randomly selected rose stems was measured each day for each treatment by weighing the cylinders containing the holding solution and a cut rose, and subtracting the weight of the cylinder and the weight of the rose. Cylinders containing only the holding solution were placed at random among the rose samples. Weighing these cylinders daily allowed correction factors for evaporation to be determined. Relative fresh weight (RFW) was measured daily by weighing cylinders with or without roses, and was expressed as a percentage of the initial fresh weight (FW) on day-1.

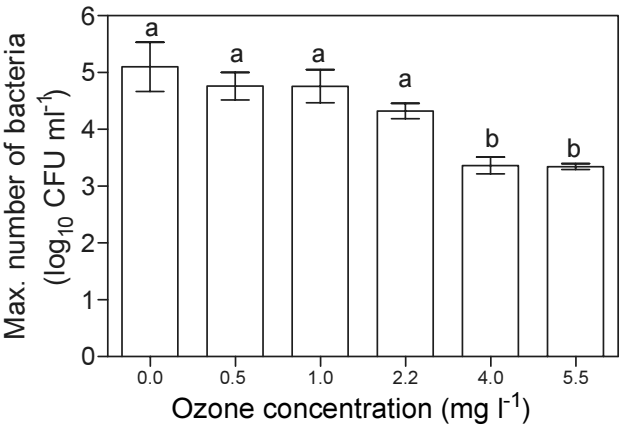

FIG. 1
Maximum number of bacteria ($\log_{10}$ CFU ml^{-1}) in the holding solution after 24 h, following treatment with ozone concentrations of 0 (deionised water), 0.5, 1.0, 2.2, 4.0, or 5.5 mg l^{-1}. Data are the means of nine replicates ± standard errors. Bars bearing a different lower-case letter are different at $P < 0.05$.

On day-5, 15 roses were selected at random per treatment and a 100 mm stem segment was cut to determine its relative water content. The segment was measured from the base of the peduncle downwards, and cut using a fresh razor-blade. Stem segments were weighed using a bench top balance (0.001 g accuracy) and placed in a vacuum flask containing 800 ml 10 mM sodium chloride for 3 h at a vacuum pressure of 10 kPa, to saturate the stem segments. After saturation, the stem segments were re-weighed, placed in paper bags and dried in an oven at 65ºC for 48 h. After drying, the stems were weighed and the relative water content (RWC) was calculated according to the equation:

$$RWC\ (\%) = [(F_w - D_w)/(S_w - D_w) \times 100]$$

where F_w was the fresh weight of the stem segment, D_w was the dry weight of the stem segment, and S_w was the weight of the stem segment after saturation.

Acid fuchsin test

For all acid fuchsin tests, roses were removed from the controlled climate chamber and placed in the laboratory at 21ºC with a PAR of 9 µmol m^{-2} s^{-1}. After 5 d of vase-life, ten stems (with leaves) per treatment were selected at random and placed in 0.25% (w/v) acid fuchsin (Sigma-Aldrich Chemicals, St. Louis, MO, USA) for 5 min at a solution depth of 5 mm. After 5 min, the stems were cut longitudinally using a sharp razor-blade and the distance travelled by the dye up the xylem was measured to determine the rate of dye uptake. Using the same stem segments, a cross-section of the stem was made 150 mm from the cut end (above the water line in the holding solutions) and observed at 40× with a dissecting light microscope. Photomicrographs were taken of the cross-sections and the percentage of vascular bundles containing dye was determined (expressed as a percentage of functional xylem vessels).

Leaf gas exchange measurement

Stomatal conductance (g_s) and the net CO_2 exchange rate (NCER) of the youngest fully-expanded leaves were measured on day-5 in 13-to-15 stems per treatment using a portable gas exchange system (LI-6400; LICOR, Lincoln, NE, USA) under the same environmental conditions as described in the *Plant material and ozone treatment* section.

Statistical analysis

All experiments were completely randomised designs (CRD). Means were compared using a Studentised *t*-test. Graph Pad Prism software (Version 4.0; GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analyses. For the water uptake and relative FW data, *t*-tests were performed to compare treatment effects on each day.

RESULTS AND DISCUSSION

Ozone concentrations of 0.5, 1.0, and 2.2 mg l^{-1} did not significantly ($P > 0.05$) reduce bacterial levels when cut roses were placed in the ozonated water, while ozone concentrations of 4.0 and 5.5 mg l^{-1} significantly ($P < 0.05$) reduced bacterial concentrations (Figure 1), without any

sign of phytotoxicity. Based on these results, an ozone concentration of 5.5 mg l^{-1} was chosen as the highest practical ozone concentration for this application. Results showed that, even with daily ozone treatments of 5.5 mg l^{-1}, no toxicity symptoms were observed on leaves, petals, or stems at any time during the experiment.

The development of bent neck, petal wilting, and/or petal discolouration was observed in cut roses stored in de-ionised water by day-3, while roses stored in ozonated water exhibited only minor petal discolouration (a slight lightening of the petals to a magenta colour) up to day-13. Thus, storage of cut roses in solutions containing 5.5 mg l^{-1} aqueous ozone significantly ($P < 0.0001$) extended vase-life to 13.0 ± 0.52 d compared to a vase life of 5.0 ± 0.35 d for roses stored in de-ionised water. These vase-life results, using aqueous ozone, were favourable compared to studies using other preservative agents employed in the cut rose industry. For example, storage of roses in slow-release chlorine dichloro-isocyanurate (DICA), hydroxyquinoline citrate (HQC), or Physan-20 increased the vase-life of cut 'Classy' roses from 6.99 d in de-ionised water to only 8.28, 9.01, or 8.41 d, respectively (Knee, 2000). Storage of cut 'Sonia' roses in 60 mg l^{-1} chlorine resulted in a vase-life of 4 d, while storage in ≥ 125 mg ml^{-1} aluminium sulphate extended vase-life to just over 8 d (van Doorn *et al.,* 1990). Also, the addition of maleic acid hydraside (MH) or sucrose to the holding solution of cut roses extended their vase-life from 4.1 d in water to 6.4 d with MH, and to 7.8 d in sucrose (Huang *et al.,* 2002).

To quantify the anti-bacterial capacity of ozone in cut rose holding solutions, bacterial growth curves were developed for de-ionised or ozonated water. Since ozonation was performed once every 24 h, the growth curves also allowed us to determine the extent to which ozone could maintain low levels of bacteria over a 24 h period. The growth curves revealed that bacterial concentrations in ozonated water remained at 0 CFU ml^{-1} for approx. 7 h, then increased to approx. 4 $\log_{10}$ CFU ml^{-1} by the end of the 24 h period, at which time all holding solutions were renewed. In contrast, the concentration of bacteria in de-ionised water began to increase from 0 h onwards, reaching approx. 6 $\log_{10}$ CFU ml^{-1} after 24 h (Figure 2). Mean bacterial concentrations over 24 h were calculated from the growth curves, and showed mean bacterial levels in the de-ionised water of 3.06 $\log_{10}$ CFU ml^{-1}, while levels in the ozonated water were only 1.16 $\log_{10}$ CFU ml^{-1}. These results corresponded well with the aesthetic appearance of roses stored in ozonated water, which showed few signs of water stress (e.g., petal or leaf wilting) throughout the experiment. The onset of senescence in cut roses is due, at least in part, to the presence of bacteria in the holding solution as demonstrated in this study and by Robinson *et al. (*2007). Therefore, it can be concluded that the primary cause of the enhanced vase-life of cut rose using ozone was a result of its antibacterial action. Many researchers have shown that high bacterial populations in the holding solutions of cut roses have a severe negative effect on vase-life (Durkin and Kuc, 1966; Burdett, 1970; de Witte and van Doorn, 1988; van Doorn *et al.,* 1995). It should be noted that although ozonation was performed every 24 h, further vase-life enhancement could be achieved with more frequent or continuous low-level ozonation.

The number of bacteria on the cut surface of each stem was also counted on day-5, as many reports have suggested that bacteria in the holding solution accumulate at the cut surface as the stalk provides a plentiful supply of nutrients. (van Doorn *et al.,* 1991; Durkin *et al.,* 1995; 2001; Put and Clerkx, 2001). The mean number of bacteria on the cut surfaces of stems stored in ozonated water (6.79 ± 0.124 $\log_{10}$ CFU g^{-1} fresh weight) was significantly ($P < 0.0001$) lower than on stems stored in de-ionised water (7.94 ± 0.067 $\log_{10}$ CFU g^{-1}). This result supports the notion that 5.5 mg l^{-1} aqueous ozone is capable of significantly reducing the level of bacteria in the cut rose holding solution.

Bacteria remain on the cut stem surface, in part, due to the hydraulic forces generated by transpiration (Dixon and Peterson, 1989; Durkin *et al.,* 1995; Put and Clerkx, 2001). This was confirmed in this study as the number of bacteria suspended in the holding solution was lower (by about 2 $\log_{10}$ CFU ml^{-1}) than the number of bacteria at the cut surface in both treatments. It was also observed that a layer of grey slime developed at the cut surfaces of rose stems stored in de-ionised water, which was absent in ozone-treated roses. Since the bacteria on the cut surface originated from the holding solution (van Doorn and de Witte, 1997), storing cut roses in aqueous ozone can prolong vase-life by reducing the number of bacteria in the holding solution and, consequently, on the cut surface of the stem.

Although the rate of water uptake during the first 3 d of the 6-d-long experiment was not significantly different between the two treatments, significantly higher rates of water uptake were seen in ozone-treated roses on days-4, -5, and -6 (Figure 3A). Relative fresh weights (RFW; Figure 3B) were not significantly different between roses in ozone-treated or de-ionised water for the first 2 d. However, the RFW of roses stored in ozonated water were significantly higher than roses stored in de-ionised water on days-3, -4, -5, and -6.

Relative water contents were measured on day-5 for 100 mm stem segments removed from directly below the peduncle. This was done since any vascular blockage present at any point in the xylem should exhibit a lower

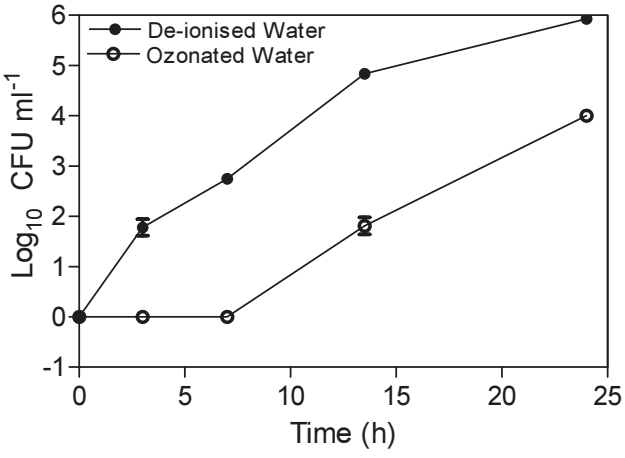

FIG. 2

Daily (24 hr) bacterial growth curves for holding solutions of cut roses containing either de-ionised or 5.5 mg l^{-1} ozonated water. Bacteria were counted each day, up to and including day-6 of the experiment which corresponded to the day after the average vase-life for roses held in de-ionised water. Data are the means of five replicates ± standard errors.

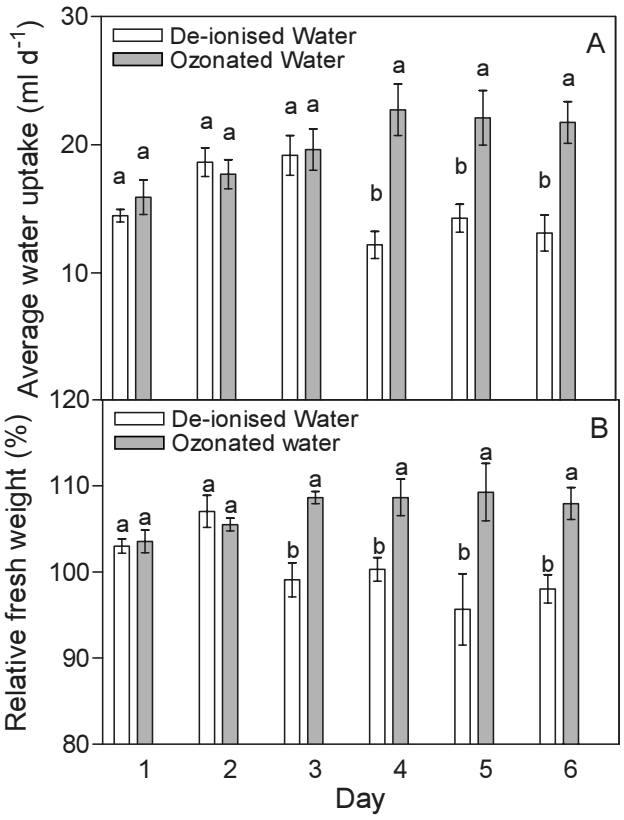

FIG. 3
Average daily water uptake (Panel A) and relative fresh weight (Panel B) during storage of cut roses stored in de-ionised water or in 5.5 mg l^{-1} ozonated water. Data are the means of six replicates ± standard errors. Bars bearing a different lower-case letter on any one day are different at $P < 0.05$.

water content near the peduncle, as this region of the stem is most sensitive to water stress. Stem segments from roses in ozonated solution showed significantly ($P < 0.0001$) higher (92 ± 0.5%) relative water contents (RWC) compared to stems stored in deionised water (85 ± 0.9%).

The presence of bacteria in the cut rose holding solution is the primary cause of vascular occlusions which impede water uptake (Robinson *et al.,* 2007). Therefore, by reducing bacterial numbers, ozone prevented the development of vascular occlusions in the xylem and, as a result, maintained higher rates of water uptake. In contrast, the premature senescence observed in roses treated with de-ionised water was probably due to bacterial infiltration into the open-ended xylem vessels, which developed into a vascular blockage (Robinson *et al.,* 2007). Acid fuchsin is a bright pink xylem-mobile dye, easily visualised using the naked eye as it travels up xylem vessels, but can be more easily seen with the aid of a light microscope. After placing cut rose stems in acid fuchsin for 5 min, those previously stored in ozonated water had a significantly ($P < 0.0001$) higher rate of acid fuchsin uptake (39.8 ± 2.31 mm min^{-1}) than roses previously stored in de-ionised water (17.1 ± 0.98 mm min^{-1}). In addition to the rate of dye uptake, the percentage of xylem vessels containing the dye 150 mm from the cut end was significantly ($P < 0.0001$) higher in roses stored in ozonated water (91 ± 2.0%) than in those kept in de-ionised water (1.3 ± 0.98%), according to photomicrographs (data not shown). A distance of 150 mm from the cut surface was chosen as this was both above the holding solution water line, and was used in all other tests. Previous studies have also shown that the deposition of gum, the formation of tyloses, and other physiological blockages developed at or above the water level (Lineberger and Steponkus, 1976).

Previous research has shown that 5.7 $\log_{10}$ CFU ml^{-1} of *Bacillus subtilis* in the holding solution of cut roses inhibited the uptake of acid fuchsin (Put and Clerkx, 2001), and that addition of anti-microbial compounds to the water increased acid fuchsin uptake (Durkin *et al.,* 2001). Low dye uptake by roses stored in de-ionised water is supported by previous research that coupled acid fuchsin uptake with hydraulic conductivity measurements and found that once bacterial concentrations reached 6 $\log_{10}$ CFU ml^{-1}, 50% of xylem vessels were blocked (Put and Jansen, 1989). If acid fuchsin was unable to move through the xylem, this indicates that water uptake was blocked and that premature senescence should result.

The stomatal conductance of leaves on roses stored in ozonated water (126 ± 5.6 mmol m^{-2} s^{-1}) was significantly ($P < 0.0001$) higher than in those stored in de-ionised water (54 ± 8.3 mmol m^{-2} s^{-1}). Mean NCER values were also significantly ($P = 0.0347$) higher in leaves of roses stored in ozonated water (6.7 ± 0.64 μmol CO_2 m^{-2} s^{-1}) compared to those stored in de-ionised water (4.9 ± 0.52 μmol CO_2 m^{-2} s^{-1}).

To adapt to conditions of water stress, stomata close to conserve water and avoid desiccation. Therefore, if bacteria caused vascular blockages in rose stems and inhibited water uptake, the stomata should close to conserve water. Harvested roses have significantly lower stomatal conductance compared to intact plants due, in part, to the numerous stress factors that occur during and after harvest. On sunny days, stomata in intact 'Sonia' and 'Cara Mia' roses typically had a g_s of 300 mmol m^{-2} s^{-1} (van Doorn and Reid, 1995), compared to g_s values of 54 ± 8.3 mmol m^{-2} s^{-1} and 126 ± 5.6 mmol m^{-2} s^{-1} for cut roses after 5 d storage in de-ionised or ozonated water, respectively. Thus, although harvesting and storage considerably reduced stomatal conductance, ozone-treated roses exhibited significantly higher stomatal conductance, even during high stress post-harvest storage, compared to roses maintained in de-ionised water.

In conclusion, storing cut 'Pascha' roses in aqueous ozone at an initial concentration of 5.5 mg l^{-1}, renewed daily, extended their vase-life almost three-fold and resulted in a prolonged and enhanced aesthetic appearance. Vase-life was enhanced by reducing the number of bacteria present in the holding solution. Thus ozone has potential as a means to extend cut flower storage-life, thereby improving efficiency and profitability for the cut flower industry. Although promising, further work is required to design a system that can ozonate commercial holding solutions more frequently, or continuously, without dramatically increasing labour and energy input costs.

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