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Ozonated Wash Water for Inhibition of *Pectobacterium carotovorum* on Carrots and the Effect on the Physiological Behaviour of Produce

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Summary

In postharvest the risk of produce losses due to mechanical injuries during harvest and handling, and to contamination with phytopathogenic microorganisms is very high. Root vegetables such as carrots are washed before packaging to remove inorganic and organic debris and to present the product appealingly. Hence, effective disinfection of the washing water is indispensable to eliminate the risk that carrots strongly loaded with microorganisms might infect previously uncontaminated tap roots. During the last few years, the addition of various sanitizers has been considered one of which is ozone. Ozone is highly bactericidic and fungicidic, and does not generate hazardous residues. On the other hand, ozone is a strong oxidant and might damage the produce. The aim of this study was to investigate the suitability of ozonated wash water for the assurance of carrots' quality and safety stored at 20 °C during 10 days. Both in solution and on carrots, ozonated water effectively inactivated *Pectobacterium carotovorum*, a bacterium which causes the soft rot. On the other hand, washing carrots with ozonated water (ozone concentration: 4 ppm) for 2 min did not affect the physiological activity of the products. Hence, ozonated water can be recommend as a rather easily applicably and efficient sanitizer for the washing of root vegetables.

Key words. emerging technology – microbiological contamination – quality of product – sanitizer

Introduction

According to the marketing standard for fruit and vegetables minimum requirements demand, among others, that the produce must be 'clean' and 'practically free of any visible foreign matter' (AID 1998). Hence, many produce need to be carefully washed to fulfil these simple claims. In addition, washing may help to reduce the product temperature and to decrease their microbial load. However, to further improve overall quality and shelf life of fresh produce several emerging physical and chemical technologies to efficiently reduce the initial microbial load are under investigation during recent years. Besides the application of heat treatment, irradiation (UV-C, γ) and peroxyacetic or lactic acid or thyme essential oil solution (VINCENTE et al. 2004; UYTTENDALE et al. 2004; RODGERS et al. 2004; WEI et al. 2005; WEISS and HAMMES 2005; ARTÉS and ALLENDE 2005), chlorine is widely used in food processing plants to sanitize fruits and vegetables (BEUCHAT et al. 1998; DELAQUIS et al. 1999, 2004; LI et al. 2001). However, it has become obvious that chlorine less effectively reduces the load of viable pathogens than initially expected (BEUCHAT and SOFOS 1999; RODGERS et al. 2004). In addition to this limited efficacy, chlorine is not flavour neutral or odourless but induces off-flavours and factitious tastes of the produce. Moreover, chlorination causes the formation of potentially mutagenic or carcinogenic organochlorine compounds, such as trihalomethanes (CHANG et al. 1988; PARISH et al. 2003) which have to be safely processed and detoxified causing additional financial efforts. Consequently, the use of chlorine is restricted by German law (LMBG 1997). Hence, the development of effective, hazard free alternative methods is of great interest.

Ozone may be a suitable sanitizer and substitute of chlorine (KHADRE et al. 2001; KIM et al. 2003). Many investigations established the bactericidal and fungicidal properties of gaseous and water-dissolved ozone (BOTT 1991; BEUCHAT 1992; RICE 2001; MARQUENIE et al. 2002; RODGERS et al. 2004). The application of ozone may be advantageous compared to chlorine treatment for many reasons. It can be applied as gas or dissolved in water. In the latter state, it can be relatively easily implemented into existing washing processes (HASSENBERG et al. 2005, 2007). Unlike chlorine ozone decomposes quickly and does not generate hazardous residues (KHADRE et al. 2001) but can even destroy pesticides (HWANG et al. 2001) and other harmful organic substance (RODGERS et al. 2004).

On the other hand, ozone is a strong oxidant which is approximately four times more reactive than chlorine (KHADRE et al. 2001) and might affect the produce metabolism (FORNEY 2003). Long term ozone treatment might reduce beneficial organic compounds contents such as biothiols or ascorbic acid (QUIANG et al. 2005), or inhibit the emission of desirable volatile esters (PÉREZ et al. 1999). Exposure to gaseous ozone may even negatively affect product physiological traits such as respiration or membrane stability, thus reducing its quality and shelf life (LIEW and PRANGE 1994). Hence, it is essential to verify that the proposed ozone treatment does not impair the treated produce.

For root vegetables especially the bacterial soft rot, caused by the bacterium *Pectobacterium carotovorum*, represents a high risk of spoilage that eventually results in a total loss of the entire harvest. Soft rot of fresh produce occurs during storage or at markets. *P. carotovorum* grows poorly at temperatures below 10 °C. At 20 °C, *P. carotovorum* is considered the most destructive soft-rotting pathogen of fruits and vegetables (Liao 2006). An infestation with *P. carotovorum* spp. results in a disintegration of the produce tissue structure. The putrefactive bacteria segregate enzymes that degrade pectic substances. Pectic substances are the most important compounds of the middle lamellae of abutting cell walls and responsible for adhesion of cells (Hachtel 2002; Lord and Mollet 2002).

In the presented study a series of independent experiments aimed to investigate the efficiency of ozonated water to inactivate *P. carotovorum* in an aqueous model system and on inoculated wash carrots. Furthermore, it evaluates the damaging effects of practically relevant concentrations of ozone dissolved in water on the produce. For this purpose, the impact of ozonated water on the relevant sensitive physiological parameters respiration and transpiration, and on the vitamin C content of the carrots were studied.

Materials and Methods

Ozone generation and measurement

Ozonated water was generated using a 'Bewazon 1' ozone generator (0.02 g O_3 min^{-1}; BWT Water Technology Ltd., Schriesheim, Germany). The temperature of the ozonated water was set to 10 °C using a thermostat 45 (Haake, Karlsruhe, Germany). Ozone concentration was measured with a LASA^R 2plus photometer applying the complementary chlorine/ozone cuvette test (Dr. Bruno Lange GmbH & Co., Düsseldorf, Germany).

Treatment of P. carotovorum *in solution*

Aliquots (10 µl) of *Pectobacterium carotovorum* (DSMZ 30168) suspensions were mixed with 10 ml of ozonated water ($c(O_3)$ = 4 ppm) or of distilled water, respectively. The resulting strain concentrations in the bacteria mixtures was $1.3 \cdot 10^5$ cfu ml^{-1}. After exposure times of 10, 20 and 30 s the mixtures were shaken with a whirler for 1 min to decompose the residual ozone. Then, aliquots of the different mixtures were plated (Mac Conkey Agar plates, Merck, Darmstadt, Germany) and incubated at 25 °C for 2 d. The experiment was performed twice.

Treatment of inoculated carrots

Freshly harvested Nantes-type bunched carrots from local producers were purchased on a fresh market. Only high quality carrots free of any visible damage or defects were used in the experiments.

Under sterile conditions in a clean bench the carrots were inoculated with *P. carotovorum* (10^8 cfu ml^{-1}) by immersing in a bacterial suspension (Ringer solution) for 30 min. Before further experimentation, carrots were allowed to dry superficially for 1 h. Inoculated (control 1), water washed (control 2), and ozonated water washed ($c(O_3)$ = 4 ppm) carrots were analysed after the treatment.

Inoculated carrots (3 carrots per treatment) were washed for 0.5, 1, 2, 5 and 10 min in ozone solution or tap water (control 2) fully immersed in an unpressurised continuous flow-through system. In case of ozonated water this system allowed exact control of ozone concentrations.

Microbiological analysis

After the respective treatment the carrots were mashed under sterile conditions. Then 10 g of the resulting slurry was added to 90 ml of Ringer solution, an aliquot diluted, plated, (plate count agar, PCA, plates, Merck, Darmstadt, Germany), incubated at 25 °C for 2 d and subsequently counted (Fig. 1).

Respiration and transpiration measurements

For the evaluation of the possible effects of the ozone treatment on produce physiology fresh carrots, purchased from a local market (see above), were used either untreated (control), washed with tap water (washed) or with ozonated water ($c(O_3)$ = 4 ppm) in a continuous system for 2 min. For each variant 3 carrots were used. The

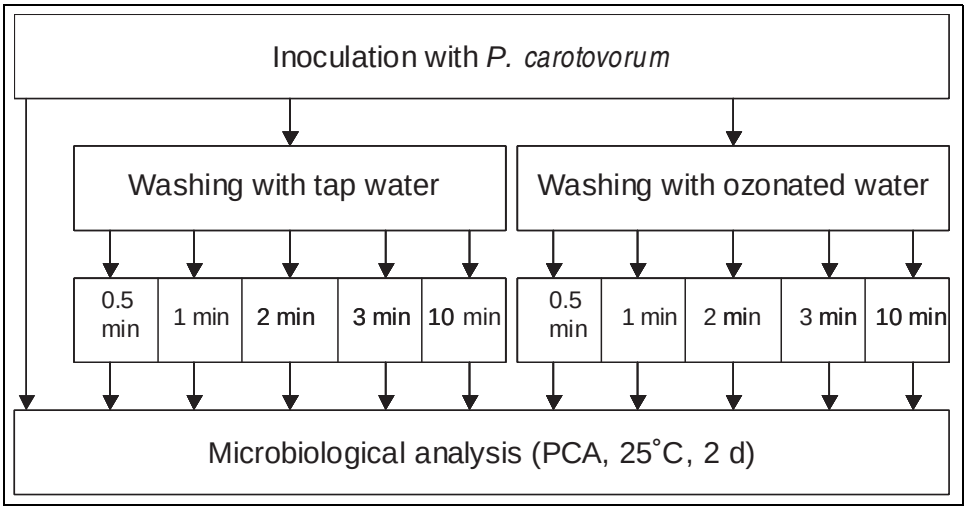


Fig. 1. Flow diagram indicating the washing variants of carrots.

carrots were dark stored at 20 °C for 10 d in nearly water vapour-saturated air. Respiratory CO_2 release of the intact carrots was determined daily in a closed system fitted with infra red sensors ($FYA600_{CO2}$, Ahlborn Mess- und Regeltechnik, Holzkirchen, Germany). From the increase in CO_2 concentration over time within a closed Perspex cylinder and the tuber surface area, respiration activity was calculated as $\mu mol_{CO2}\ cm^{-2}\ h^{-1}$. Transpiration water losses (mmol $m^{-2}\ s^{-1}$) of each carrot were repeatedly determined by consecutive weighing of the carrots before and after free transpiration at free convection under controlled environmental conditions (T_{air} = 22.1±0.9 °C; $VPD_{carrot-air}$ = 11.7±1.7 hPa) for 0.5 h. From the transpiration rates and the respective data of air and tuber temperatures and humidity of the surrounding air, total tuber conductance for water vapour transfer (g_{H2O}) was calculated (Von Willert et al. 1995).

Analysis of vitamin C

For the determination of the vitamin C content each carrot was cut longitudinal into halves. The vitamin C content of one half was determined after washing with 2.0 L tap water for 2 min, the second half was washed for 2 min in 2.0 L ozonated water ($c(O_3)$ = 4 ppm). Also water washed but not inoculated carrots were analysed as a control. A juice extractor (Multipress MP 32, Type 4232, Braun GmbH, Kronberg/Taunus, Germany) was used to obtain the tissue juice. The samples were filtered und diluted with distilled water (1:2) before the vitamin C content was measured using a 'Reflectoquant' test set (Merck, Darmstadt, Germany). Each experiment was performed five times.

Statistical Analysis

Data were statistically analysed (ANOVA) with WinSTAT (R. Fitch Software, Bad Krozingen, Germany). Significant differences were determined by the Duncan's multiple range test ($P<0.05$).

Results

Effect of ozonated water on P. carotovorum *in solution and on carrots*

Treating the *P. carotovorum* suspensions with ozonated water ($c(O_3)$ = 4 ppm) for 10, 20, and 30 s, completely inactivated the bacteria. No colonies growth was found on the agar plates. This clearly indicates that *P. carotovorum* is sensitive to ozone. Even a 10-second treatment with an ozone concentration of 4 ppm reduces the bacterial content from $1.3{\cdot}10^5$ cfu ml^{-1} to less than the limit of the detection method (10^2 cfu ml^{-1}).

The total bacterial count on untreated fresh carrots was found to be 3.5 10^3 cfu g^{-1}±2.3 10^3 cfu g^{-1}. In contrast, the content of *P. carotovorum* was always less than 10^2 cfu g^{-1}. On inoculated control carrots, the bacterial count of *P. carotovorum* was initially $7{\cdot}10^6$ cfu g^{-1}. Within the initial two minutes of ozone treatment the count of *P. carotovorum* decreased to $1.8{\cdot}10^5$ cfu g^{-1}, which is a 1.5 log reduction. Any additional extension of the contact time did not further decrease the number of bacteria. Simply washing the inoculated carrots with tap water also reduced the bacterial load independently of the washing time. However, the average reduction that was achieved by washing with tap water was only half a log. Hence, compared to tap water, washing intact carrots with ozonated water for at least 2 min much more effectively (one log) reduces the contamination with *P. carotovorum* (Fig. 2).

Effects of ozonated water on respiration, transpiration and vitamin C content

Due to the high air humidity during storage, initial relative mass losses were low (5.8±2.3 %) and increased by approximately 3.8 times to 21.9±1.1 % after 9 days of storage (Fig. 3A). However, these changes applied for all carrots, irrespective of the treatment.

The same was true for tuber conductance, which declined significantly by nearly 20 % during the very initial

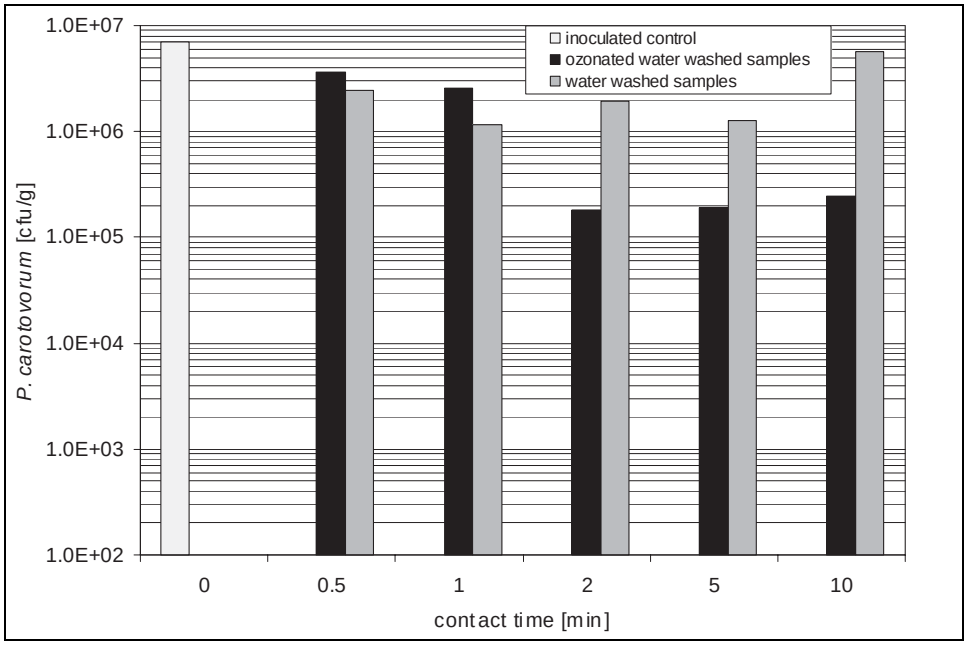


Fig. 2. Counts (shown is the average of 3 independent samples) of *P. carotovorum* on inoculated carrots and on carrots after washing with tap water and ozonated water.

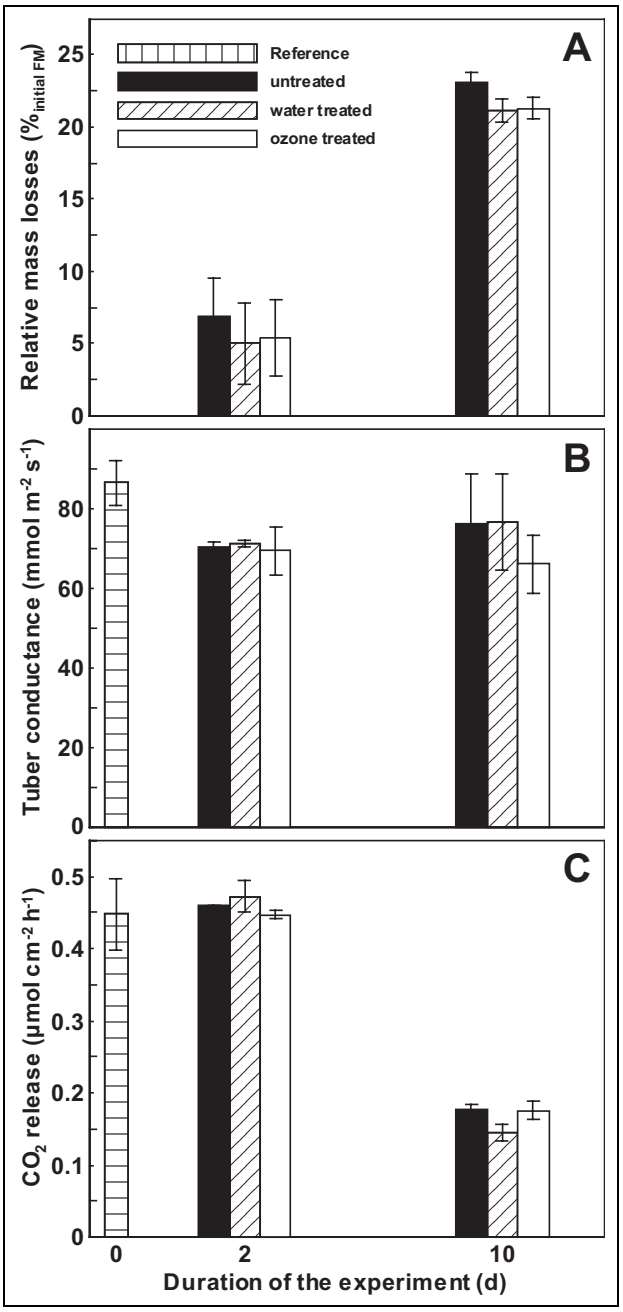


Fig. 3. Means (±sd) of (A) relative mass losses (related to the initial fresh mass), (B) tuber conductance and (C) tuber CO_2 release of fresh carrot tubers and carrots stored at 20 °C and nearly water saturated air for 2 and 10 d, respectively. Before storage carrots were either kept untreated (dotted bars), washed with water (hatched bars) or washed with ozonated water ($c(O_3)$ = 4 ppm).

storage (Fig. 3B) and remained constant at approximately 71 mmol m^{-2} s^{-1} (±9 mmol m^{-2} s^{-1}). In contrast, respirational activity as indicated by the CO_2 release rates declined only after prolonged storage, again in all carrots irrespective of the treatment (Fig. 3C).

In fresh untreated carrots the vitamin C content ranged between 93 and 138 mg l^{-1} yielding an average (± sd) of 113±18 mg l^{-1}. As expected washing with tap water did not at all affect the ascorbic acid content (114±19 mg l^{-1}). Although the vitamin C content of the samples washed with ozonated water was slightly reduced (106±14 mg l^{-1}) the changes were not significant.

Discussion

In the aqueous suspension of *P. carotovorum* the addition of 4 ppm ozone effectively and rapidly reduced the concentration of viable bacteria. Even the shortest exposure time of 10 s decreased counts of *P. carotovorum* by 3 log units. On the other hand, on carrots, artificially infected by *P. carotovorum*, the success of ozone treatment was somewhat lower. Here, the microbial load declined by only 1.5 log units after a treatment of 2 min and any extension of the contact time did not result in a further reduction of *P. carotovorum*. Hence, the presented results reflect findings on other foodborne spoilage and pathogenic bacteria of the genera *Pseudomonas*, *Escherichia*, *Leuconostoc* and *Listeria* which showed a very rapid and very effective ozone inactivation in an aqueous solution (Kim and Yousef 2000; Rodgers et al. 2004). Although still very efficient, the inactivation efficacy of water-solved ozone was found to be significantly lower on artificially inoculated apples, lettuce, strawberries, cantaloupe (Rodgers et al. 2004) or cabbage (Fisher et al. 2000). Surprisingly, aqueous ozone seemed to only be slightly more efficient than water to reduce total aerobic plate counts on pre-cut green peppers (Ketteringham et al. 2006). These authors concluded that whole pepper might be more suitable for treatment than cut fruits.

The exact reasons for the increased resistance of produce-borne bacteria against inactivation by washing with ozone solution as in the case of *P. carotovorum* on carrots is at present unknown. One explanation may be an extensive clumping of cells, similar to the formation of aggregations of *Bacillus globigii* (Currier et al. 2001) or the formation of biofilms by bacteria such as *Pseudomonas* spp. (Madigan et al. 2000). This may result in a largely reduced contact surface of the reactive ozone thus reducing its chance to penetrate and react with components of the cell envelope, or intracellular membranes and enzymes (Khadre et al. 2001). On the other hand, the accessibility of the microorganisms to the action of the sanitizer may be reduced due to the close attachment of the bacteria to the carrot surface (Achen and Yousef 2001).

The high reactivity of ozone with organic compounds (Hwang et al. 2001) contaminating carrot tap roots may also contribute to the reduction of ozone efficiency. Ketteringham et al. (2006) assumed that organic matter leached from cut pepper slices produce a high ozone demand solution thus reducing the effectiveness of a aqueous ozone. Indeed, direct reaction of solved ozone with unsaturated organic compounds may lead to a pronounced ozone consumption and hence rapid reduction of effective ozone concentration (Hassenberg et al. 2007). Hence, continuous control of the constant effective ozone concentration e. g. by continuous addition of ozone to the wash water may be more effective (Achen and Yousef 2001).

Nevertheless, the presented results confirm that ozone may be a helpful additive to control the risk of *P. carotovorum* infection of carrots during washing. Furthermore,

the high sensitivity of this spoilage bacterium to ozone treatment in solution implicates the use of this sanitizer for a successful decontamination of the washing facility which should further reduce the risk of spoilage of washed carrots.

There have been some reports that ozone is not a safe sanitizer (Liew and Prange 1994; Forney 2003; Quiang et al. 2005). It has been shown during long-term exposure of strawberries to gaseous ozone that it may have detrimental effects on fruit aroma and emission of volatiles (Pérez et al. 1999). Prolonged aqueous ozone treatment of produce such as spinach, cucumber, asparagus, green bean or red pepper may reduce their contents of biothiols (Quiang et al. 2005). Ozonation has also been reported to significantly decrease the ascorbic acid content in potatoes, cabbage and carrots (Enshina et al. 1989 as cited by Quiang et al. 2005). Furthermore, Liew and Prange (1994) found that exposing carrots 28 d for 8 h daily to gaseous ozone of up to 60 $\mu l\ l^{-1}$, besides discoloration, resulted in significantly increased respiration and decreased membrane stability.

None of these negative effects could be found in our experiments. Despite the fact that rates of ozone penetration into non-stomatous surface tissues are low (c. f. Padu et al. 2005) ozone certainly passes peridermal carrots surface tissue as shown by Liew and Prange (1994). It then rapidly dissolves in the apoplast where it may generate various reactive oxygen species (Pinghua et al. 2006). Primary targets of direct and indirect ozone activity are the unsaturated lipids and proteins of the cell membranes of the exposed cells (Leipner et al. 2001; Oksanen et al. 2003). This, in turn may initially lead to a substantial disturbance of membrane function and semipermeability (Tokarska-Schlattner et al. 1997). Hence, any direct destruction or disturbance of the peridermal surface tissue which was directly exposed to ozone should have significantly increased the water vapour conductance of the treated carrots. In contrast, washing with high concentration of ozone did not affect total tuber conductance for water vapour transfer even irrespective of storage time. This indicates that washing of carrots may not cause any membrane damage.

On the other hand, Liew and Prange (1994) reported that relative electrolytic leakage in carrot peels increased with the ozone concentration during long-term storage of 28 d. Electrolytic leakage is clearly a sensitive indicator of membrane destruction. This specific ozone effect was also accompanied by an enhanced respiration in the above mentioned study. Increased respiratory activity can be attributed to a metabolic disturbance or, more probably, to a rising energy and substrate demand for repair processes of ozone caused internal injuries (Liew and Prange 1994). In our investigation, ozone treatment did not affect respiration. Hence, no metabolic disturbance or internal injuries occurred during a short-term peak exposure by washing the carrot with ozonated water. Thus, ozonated water may not be harmful to the tubers.

From many investigations on the ozone effects on various plants (Tokarska-Schlattner et al. 1997; Oksanen et al. 2003; Kangasjärvi et al. 2005; Pinghua et al. 2006) it became obvious that long-term chronic exposure to high concentrations of gaseous ozone may override the very effective apoplastic detoxification by reduced ascorbic acid (Noctor and Foyer 1998, Plöchl et al. 2000). Only under ascorbic acid deficiency ozone may be able to directly act with the internal cellular components (Moldau et al. 1998). And, vice versa, it seems probable that chronic ozone effects may result in a reduction of the ascorbic acid content. On the other hand, it has been found that chronic ozone treatment significantly increases the ascorbic content of strawberries (Pérez et al. 1999) and tomatoes (Aguayo et al. 2006). Non of these effects occurred in ozonated-water washed carrots. Hence, it seems reasonable that short term exposure to high concentration of water-solved ozone does not affect tuber metabolism.

In conclusion, the presented results clearly indicate that the short term disinfection of carrots by washing with ozonated water using ozone concentrations of up to 4 ppm does not negatively affect the physiological efficiency and hence the inner quality of the produce. Although the efficacy of the proposed treatment in inactivating *P. carotovorum* on artificially inoculated carrots still needs to be improved, the reduction of suspended bacteria by ozonating the washing water was nearly complete. An increase of the applied ozone concentration e. g. due to lower wash water temperatures, or higher working pressure in the reaction chamber, as well as implementation of static mixing systems is suggested and should result in further optimisation of the treatment. In addition, washing of fresh produce with ozonated water might also reduce the potential of infection with human pathogens which is described to be connected with bacterial soft rot (Wells and Butterfield 1997, 1999). However, the effect of ozone on synergistic and antagonistic interactions between soft rot, human pathogens and other, non-pathogenic microorganisms have to be investigated in further studies.

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