The Potential of Gaseous Chlorine Dioxide for the Control of Citrus Postharvest Stem-End Rot Caused by *Lasiodiplodia theobromae*

Tian Zhong,^{1,2} Jiuxu Zhang,² Xiuxiu Sun,³ Jingjing Kou,² Zhike Zhang,² Jinhe Bai,³ and Mark A. Ritenour^{2,†}

¹ Faculty of Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau 999078, China

² Indian River Research and Education Center, University of Florida, Fort Pierce, FL 34945, U.S.A.

³ Horticultural Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fort Pierce, FL 34945, U.S.A.

Abstract

The focus of this study was to develop technologies using chlorine dioxide (ClO₂) gas to control postharvest stem-end rot of citrus caused by *Lasiodiplodia theobromae*. Mycelial growth of *L. theobromae* on potato dextrose agar (PDA) plugs was completely inhibited by a 24-h ClO₂ exposure provided by 0.5 g of solid ClO₂ generating granular mixture in a 7.7-liter sealed container. In vivo experiments were conducted on artificially inoculated Tango and naturally infected U.S. Early Pride mandarins. When ClO₂ treatments were initiated 0 to 6 h after inoculation, decay development was significantly reduced as compared with the control, and higher ClO₂ doses were more effective. A ClO₂ treatment (using 3 g of generating mixture per 7.7-liter sealed container) administered 0 h after inoculation resulted in 17.6% Diplodia stem-end rot incidence compared with 95.6% in the control, whereas the same treatment administered 24 h after inoculation was much less effective, resulting in 63.0% incidence compared with 85.4% in the

control. Diplodia stem-end rot incidence of naturally infected fruit after using 6 or 9 g of generating mixture per 24-liter sealed box was 23.8 or 25.7%, respectively, compared with 47.9% for control fruit. The ClO₂ treatments had no negative effects on fruit quality characteristics including weight loss, firmness, puncture resistance, titratable acids (TAs), total soluble solids (TSSs), and rind color. Albedo pH at wounds was significantly reduced from 6.0 to 4.8 by the ClO₂ treatments, whereas undamaged albedo remained at 5.8. In addition, no visible physiologic defects, such as peel browning and bleaching, were observed on ClO₂-treated fruit. These results indicate that ClO₂ gas has the potential to be developed as a component of an integrated citrus postharvest decay control system to minimize fruit losses.

Keywords: chlorine dioxide, citrus, *Diplodia natalensis*, *Lasiodiplodia theobromae*, postharvest, stem-end rot

Citrus is a popular and economically important horticultural crop that is widely cultivated in tropical and subtropical regions in the world. Citrus is regarded a universal fruit, with production in >100 countries spanning six continents (Saunt 1990). Citrus fruits are negatively affected by many factors from production to final consumption. Postharvest diseases can have a significant impact on fruit quality, marketing values, and economic returns.

Postharvest losses are more costly than production losses because of added harvesting and postharvest handling costs invested in the fruit. Furthermore, quality problems can reduce consumer confidence and thus reduce repeated buying. Several postharvest diseases such as green mold (caused by *Penicillium digitatum*), blue mold (caused by *Penicillium italicum*), stem-end rots (caused by *Lasiodiplodia theobromae* and *Phomopsis citri*), sour rot (caused by *Galactomyces citri-aurantii*), and anthracnose (caused by *Colletotrichum gloeosporioides*) occur in Florida, but stem-end rot caused by *L. theobromae* is the most common problem in early-season citrus (Ismail and Zhang 2004; Zhang 2007). Decay incidence often reaches 13 to 42% when fruits are not treated with a proper fungicide and/or placed in refrigeration (Smilanick et al. 2006). Stem-end rot caused by *L. theobromae* was historically called Diplodia stem-end rot, and we shall continue to use that name in this report. *L. theobromae* is a facultative pathogen that completes its life cycle on dead twigs of citrus trees in the grove. Postharvest stem-end rot arises from the latent infection of citrus fruit button (i.e., calyx

and disk) tissues by *L. theobromae* in the field (Zhang 2007). It can be a severe problem, especially when fruits are ethylene-degreenned, a practice that is often done on early-season fruit to enhance peel coloration for improved marketing (Barmore and Brown 1985; Brown 1975).

Furthermore, an increased Diplodia stem-end rot incidence has been observed on Huanglongbing (HLB)-affected fruit in Florida (Zhao et al. 2015). This may be partially because of the increased amount of weakened and dead wood in HLB-affected trees, which promotes growth and sporulation of *L. theobromae* (Ritenour et al. 2016). Therefore, there is a serious need for effective control measures for Diplodia stem-end rot of Florida citrus.

In an effort to manage postharvest decay and extend the shelf life of fresh citrus, a variety of approaches have been used, such as the application of the fungicides thiabendazole (TBZ), imazalil, and sodium-o-phenylphenate (SOPP) and the sanitizers ozone, hydrogen peroxide, peracetic acid, chlorine, and chlorine dioxide (ClO₂) (Allende et al. 2006; Beuchat 1996; Ritenour et al. 2016). ClO₂ is approved by the U.S. Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA) for application in water treatments (James 2006) and is a strong oxidizing agent with approximately 2.5 times higher activity than chlorine and with a broad biocidal effectiveness (Benarde et al. 1965). In most cases, ClO₂ is used in gaseous or aqueous forms. Gaseous ClO₂ has been proved to be more effective in deactivating microorganisms than aqueous ClO₂ because of its superior ability to reach cavities and to penetrate produce with uneven surfaces (Du et al. 2002). Moreover, the use of gaseous ClO₂ has become more attractive because it minimizes changes in aroma and taste of the treated commodity (Couri et al. 1982).

Although the efficacy of gaseous ClO₂ to control several microorganisms in different fruits and vegetables has been documented (Keskinen et al. 2009; Mahovic et al. 2007; Sun et al. 2014, 2017b), little research has focused on applications for control of postharvest decays of citrus, including Diplodia stem-end rot control. Our goal was to determine whether gaseous ClO₂ treatments have the potential to manage postharvest Diplodia stem-end rot. The specific objectives of this study were to (i) determine the effect of ClO₂ gas against

[†]Corresponding author: M. A. Ritenour; ritenour@ufl.edu

Funding: This work was supported by State Scholarship Fund of China Scholarship Council grant 201708440438 and Florida Citrus Packers through a U.S. Department of Agriculture Technical Assistance for Specialty Crops grant TASC-2019-02.

The author(s) declare no conflict of interest.

Accepted for publication 30 April 2021.

L. theobromae in vitro, (ii) evaluate the effect of ClO₂ gas on fruit decay development, and (iii) evaluate the effects of ClO₂ gas treatment on the quality of treated mandarins.

Materials and Methods

Fruit and fungal pathogen. Tango fruits were obtained from a 3-year-old commercial grove in Saint Lucie County, Florida, and U.S. Early Pride fruits were harvested from 5-year-old trees at the U.S. Department of Agriculture–U.S. Horticulture Research farm in Fort Pierce, Florida. Both mandarins were still green, with a slightly lower total soluble solid (TSS; 8.7) than the minimum maturity standards (9.0) when harvested. Fruits were transported immediately after harvest to the University of Florida–Indian River Research and Education Center (UF/IRREC) in Fort Pierce and stored for 2 days at 10°C before the experiments. All fruits were free of defects and similar in size (average diameter approximately 80 mm) and color. Extra fruits from each harvest that were not needed for the first experiment were stored at 10°C and used to repeat the same experiments (storage length approximately 5 days). Initial fruit quality parameters, including color, firmness, Brix, and acid content of U.S. Early Pride mandarin were measured before each experiment. An isolate of *L. theobromae* was obtained from citrus fruit (U.S. Early Pride) with stem-end rot at UF/IRREC and used in the study. The isolate was hyphal tipped for further purification. The identification of the isolate was further confirmed by PCR of the fungal DNA with the primers to amplify the sequences of the partial internal transcriber spacer (ITS) and translation elongation factor 1- α (EF1- α) regions. The isolate was maintained on potato dextrose agar (PDA) in test tubes at 4°C.

Chlorine dioxide gas generation and concentration measurement. ClO₂ gas was generated by mixing two parts of dry granules (ICA TriNova, Newnan, GA) consisting of a zeolite carrier impregnated with sodium chlorite and an acid activator (FeCl₃) in a proprietary formulation. Upon mixing, ClO₂ gas is released in a predictable and repeatable manner (Smith et al. 2015). Actual ClO₂ concentrations were measured with a PortaSens II ClO₂ gas detector (Analytical Technology, Collegeville, PA).

In vitro activity of ClO₂ against mycelial growth of *L. theobromae*. The in vitro activity of ClO₂ against *L. theobromae* was determined using a chamber method described by Sun et al. (2014) with minor changes. As shown in Figure 1A, airtight polypropylene containers with a 7.7-liter volume each were surface sterilized with 70% alcohol and used to provide a sealed environment for ClO₂ exposure. *L. theobromae* was grown on PDA at 25°C for 3 days. For each treatment, a 6-cm-diameter petri dish containing 10

fungal PDA plugs (each 0.5 cm in diameter) from the edge of the fungal colony was placed on the bottom of the container. Before the container was sealed, a small sachet (6 × 6 cm) made of semipermeable fiber film containing mixed ClO₂-generating granules was attached to the inner side of the top lid with double-sided tape. Doses of 0, 0.25, 0.50, 0.75, 1.00, 1.25, or 1.50 g of mixed granules were applied to generate seven different concentrations of ClO₂ in the containers. After a 24-h exposure at 20°C, the containers were opened, the petri dishes with fungal PDA plugs were removed and incubated at 20°C for an additional 24 h, and mycelial growth was measured. The experiment was repeated three times.

Efficacy of ClO₂ treatments for control of *Diplodia* stem-end rot of inoculated mandarins. Based on the results of in vitro studies, four separate tests were conducted on Tango mandarins (Fig. 1B). In each test, a total of 225 fruits were randomly arranged into five groups: natural control (noninoculated fruit without ClO₂ treatment), inoculated control (inoculated fruit without ClO₂ treatment), single dose (inoculated fruit treated with 1 g of ClO₂-generating granular mixture), double dose (inoculated fruit treated with 2 g of granular mixture), and triple dose (inoculated fruit treated with 3 g of granular mixture). For each group, the 45 fruits were arranged into three replicates of 15 fruits (approximately 2,225 ± 7.3 g) each. Except for the natural control fruit group, all other fruits were inoculated by placing a PDA plug (0.5 cm in diameter) with *L. theobromae* mycelia at the stem-end cavity after the button tissues (disk and calyx) were removed with a sterile steel probe. The inoculated fruits and a petri dish containing 10 fungal PDA plugs were immediately (0 h, test 1) placed into the 7.7-liter container and exposed to ClO₂. The containers were sealed to prevent the gaseous ClO₂ from diffusing out. The detailed steps of ClO₂ treatment (sachet attaching, container sealing, and ClO₂ concentration monitoring) were the same as for the in vitro experiment described above. After 24-h exposure of the fruit to ClO₂ at 20°C, the containers were opened, and the petri dishes with fungal PDA plugs were removed, incubated for an additional 24 h at 20°C, and evaluated for fungal growth. Treated fruits were transferred to incubators at 30°C and 90% RH, and *Diplodia* stem-end rot incidences were recorded after 2 days. In tests 2, 3, and 4, inoculated fruits were preincubated at 30°C and 90% RH for 6, 12, or 24 h, respectively, before ClO₂ treatment to evaluate postinfection efficacies of ClO₂ treatments. The experiment was repeated.

Efficacy of ClO₂ treatments for control of *Diplodia* stem-end rot of naturally infected mandarins. The experiment was conducted with U.S. Early Pride mandarins in unsealed polypropylene boxes (24 liters in volume) immediately after harvest. For each

Fig. 1. A, ClO₂ exposure chamber for fungal potato dextrose agar plugs and fruit. B, Schematic of experiments with *Lasiodiplodia theobromae*-inoculated fruit.

treatment, 105 fruits were randomly divided into three replicates of 35 fruits each per box (average weight approximately 5,000 g). A ClO₂ sachet (6 × 6 cm) was attached to the inner surface of the top lid of each box with double-sided tape. The ClO₂ doses were as follows: (1) control, no ClO₂ treatment; (2) 3 g of ClO₂ generating granular mixture; (3) 6 g of granular mixture; and (4) 9 g of granular mixture. After treatment at 20°C for 24 h, the fruits were degreened with 5 µl/liter of ethylene at 30°C and 90% RH for 48 h, incubated at 20°C and 75% RH for up to 2 weeks, and then evaluated for *Diplodia* stem-end rot development. The decay incidence was calculated as $n_{\text{decay}}/n \times 100\%$, where n_{decay} is the number of decayed fruits and n is the total number of fruit assessed in each replicate. This experiment was repeated three times.

Fruit quality evaluation. The effects of ClO₂ treatments on fruit weight loss were evaluated using U.S. Early Pride mandarins from the naturally infected fruit experiments. All 35 fruits in each of three replicates of each treatment were numbered and individually weighed before ClO₂ exposure. After 24 h of ClO₂ treatment, 48 h of ethylene degreening (5 µl/liter), and an additional 2 weeks of incubation at 20°C, six fruits without decay were chosen from each replicate and weighed individually to obtain the final fruit weight loss values. The average weight loss of each treatment (three replicates) was expressed as the percentage loss of the initial total weight of the 18 selected fruits.

For determination of fruit firmness and peel puncture resistance, 18 extra U.S. Early Pride mandarins at the beginning of the naturally infected fruit experiments were used to determine the initial values. These were randomly divided into three replicates with six fruits each. At the end of the experiments, the six fruits used to measure weight loss as described above were also used to obtain final fruit firmness and peel puncture resistance values. Firmness was measured with a TA-XT2i texture analyzer (Stable Micro Systems, Godalming, United Kingdom) equipped with a 60-mm-diameter flat plate. The methods were adapted from those of Chen et al. (2016) with minor modifications. The fruits, were pressed by a flat plate at the equatorial area with a loading displacement of 12 mm. The pretest, test, and posttest speeds all were set at 1 mm/s, and the trigger force was at 1 N. Peel puncture resistance was measured using the same instrument as above with a 2-mm-diameter cylindrical probe. The other parameters were set as follows: pretest and test speeds were both at 2 mm/s, posttest speed was at 6 mm/s, and the probe travel distance after touching the fruit surface was 15 mm. The trigger force was 0.25 N. Each fruit was punctured at three random locations along the fruit equatorial region.

After performance of the firmness and peel resistance tests, including the 18 extra fruits described earlier, the same fruits were used for TSS and titratable acidity (TA) tests. Each of six fruits from each replicate of the experiment treatment were peeled and homogenized together as a new replicate. The homogenates were centrifuged at 4,000 × *g* for 10 min, and the supernatant was collected and used for TSS and TA analysis. TSS content (in percentage) of the supernatant was determined with a PAL-1 pocket refractometer (Atago, Japan). Titratable acidity (expressed as percentage citric acid) was analyzed by titrating a 40-ml supernatant aliquot to pH 8.3 using a DL12 titrator (Mettler, Hightstown, NJ).

The effect of ClO₂ on fruit rind color was evaluated using U.S. Early Pride mandarins in airtight polypropylene containers (7.7 liters). A total of 225 fruits were randomly divided into five groups with three replicates (boxes) of 15 fruits in each box (average weight approximately 2,000 g). A small sachet (6 × 6 cm) containing mixed ClO₂-generating granules was attached to the inner surface of the top lid of each box with double-sided tape. Five ClO₂ doses, 0, 1, 3, 6, or 9 g of ClO₂-generating granules per container, were used in this study. Before ClO₂ treatment, three approximately 3-cm-diameter circles were drawn at equidistant locations around each fruit equator (12 fruits per treatment) with a permanent marker to ensure that consecutive color measurements were made at the same position each time. Initial rind color was measured before (0 h) and 24 h after ClO₂ treatment with a colorimeter (CR400; Minolta). Measured parameters included lightness (*L**), chroma (*C**), and hue angle (*h*^o).

Fruit rind pH was measured on the same fruit used to evaluate peel color based on the method described by Montesinos-Herrero et al. (2011). Before ClO₂ treatment, one random fruit from each treatment was wounded at three equidistant points around the equator by cutting the flavedo with a knife tip, leaving exposed albedo tissue 5 mm in diameter and 2 mm in depth. A volume of 30 µl of distilled water was added to each wound, and pH was determined using a pH meter with a 2-mm-diameter combination electrode (Waterproof pHTestr 3+ Double Junction, Oakton Instruments, Vernon Hills, IL). The pH was measured immediately before the ClO₂ treatments and afterward to obtain initial and final values, respectively. Three fresh wounds were also made on the same fruit immediately after ClO₂ treatment, and the pH of fresh wounds was determined.

Data analysis. Data were analyzed with SPSS version 17.0 software (Experian QAS, Boston, MA) for one-way analysis of variance (ANOVA). When significant differences ($P < 0.05$) were found, the treatment means were compared using Duncan's multiple range test ($P < 0.05$). For repeated experiments, homogeneity of variance was tested by Bartlett's test, and the similar results were combined before data analysis.

Results

Chlorine dioxide generation and concentration changes during experiments. Generally, ClO₂ concentrations gradually declined during the 24-h treatment (Fig. 2). The same dose of ClO₂-generating granular mixture resulted in different ClO₂ concentrations within the containers depending on the presence of fruit. Containers with 0.5, 1.0, or 1.5 g of granular mixture and sealed for 2 h accumulated average ClO₂ concentrations of 43.7, 65.8, or 86.9 ppm, respectively, but only 12.5, 26.5, or 39.2 ppm, respectively, when 2,225 g of fruits were also sealed inside. At the end of the treatment (24 h after initiation), the average concentrations for 0.5, 1.0, or 2.0 g of ClO₂-generating granular mixture treatments without fruit were 19.5, 25.9, or 31.5 ppm, respectively, whereas those with fruit all decreased to near zero.

In vitro activity of ClO₂ against mycelial growth of *L. theobromae*. The zone outlined in green in Figure 3 represents ClO₂ treatments that completely inhibited the mycelial growth of *L. theobromae* on PDA plugs. No mycelial growth was observed from plugs treated with 0.5 g or more of ClO₂-generating granular mixture without fruit, whereas at least 2.0 g of the granular mixture was needed to completely inhibit fungal mycelial growth on plugs in the experiments when fruit was included. As shown in Figures 2 and 3, a 24-h exposure with an initial ClO₂ concentration above 43.7 ppm and a final concentration above 19.5 ppm completely suppressed *L. theobromae* growth from PDA plugs. However, a dose of 1.5 g of ClO₂-generating granular mixture did not completely prevent fungal growth in the experiment with fruit (Fig. 3).

Fig. 2. Time-course profiles of ClO₂ concentrations in experiments without and with inoculated Tango mandarins in 7.7-liter sealed containers at 20°C. The average total weight of fruit per container was 2,225 ± 7.3 g.

Efficacy of ClO₂ for control of Diplodia stem-end rot of inoculated mandarins. No disease developed on natural control fruit that were not inoculated and not treated during incubation in all tests (Table 1). Inoculated control fruit that were not treated with ClO₂ showed a range of Diplodia stem-end rot incidences from 82.7 to 95.6% in the four experiments. ClO₂ treatments reduced Diplodia stem-end rot incidences compared with the inoculated controls in all tests ($P = 0.05$), demonstrating the effectiveness of ClO₂ exposure in suppression of decay development under test conditions. The incidence of 17.7% was observed on the fruit treated with 3 g of ClO₂-generating mixture immediately (0 h) after inoculation, compared with 95.6% of decay incidence of inoculated control fruit in test 1. Test 2 showed that the increase of ClO₂-generating mixture from 1 to 3 g per container resulted in the decrease of Diplodia stem-rot incidence on the inoculated fruit incubated for 6 h before ClO₂ treatment. However, the increase of ClO₂-generating granular mixture rates from 1 to 3 g per container did not similarly reduce decay incidences in tests 3 and 4, in which the fruits were inoculated for 12 h or 24 h before ClO₂ treatment. In addition, Table 1 also shows that inoculated fruit incubated for 0 or 6 h (test 1 and 2) before treatment with ClO₂-generating granular mixture at 3 g per container had significantly lower ($P = 0.05$) decay incidences than did those incubated for 12 or 24 h (tests 3 and 4).

Efficacy of ClO₂ for control of Diplodia stem-end rot of naturally infected mandarins. Experiments with naturally infected fruit were repeated three times with similar results according to the Bartlett's test of homogeneity of variance and were combined (Fig. 4). The treatment with 3 g of ClO₂-generating granular mixture did not significantly reduce Diplodia decay incidence, whereas the 6-g and 9-g doses significantly reduced decay incidence to 23.8 and 25.7%, respectively, compared with the control incidence of 47.9%. There was no significant difference in Diplodia stem-end rot incidence between the 6-g and 9-g treatments.

Effects of ClO₂ exposure on mandarin quality. The firmness and TSS content of all fruit declined after 2 weeks of storage (Table 2). No significant treatment differences were found in fruit weight loss, firmness, and TSS. However, peel puncture resistance of control fruit and fruit treated with 3 g of ClO₂-generating granular mixture declined significantly after storage, whereas the 6- and 9-g treatments did not. The TA content of control fruit decreased significantly after 2 weeks of incubation, whereas ClO₂-treated fruit showed no significant changes. There were no significant differences in fruit peel color (L^* , C^* , and h°) as a result of the ClO₂ treatments (data not shown). Albedo pH before the ClO₂ treatments were 5.9 to 6.0 (Table 3). After ClO₂ treatments, the albedo pH of original wounds decreased with increasing ClO₂ treatment levels, down to a

pH of 4.8 at the highest level (9 g of ClO₂-generating mixture). However, the pH of albedo from fresh wounds declined only slightly to 5.8, with no effects from the different ClO₂ treatments. No visible physiologic defects, such as peel browning and bleaching, were observed on treated fruit.

Discussion

In the current study, the potential of gaseous ClO₂ for the postharvest control of Diplodia stem-end rot on early-season harvested mandarin citrus was evaluated in vitro and on fruit. *L. theobromae* mycelial growth was completely inhibited with 0.5 g or more of ClO₂-releasing product in a sealed 7.7-liter container. ClO₂ treatments significantly reduced Diplodia stem-end rot incidences on inoculated and naturally infected mandarins compared with controls. Fruit inoculation tests also showed that the decay reduction efficacies were related to ClO₂ concentrations and duration of pathogen postinfection before ClO₂ treatment. In addition, no visible fruit injuries such as peel browning and bleaching were observed on ClO₂-treated fruit.

In our current study, it was observed that ClO₂ could be significantly absorbed by citrus fruit (Fig. 2). This should be an important factor to be considered when determining the ClO₂ treatment rates for controlling citrus fruit decay. According to previously reported

Table 1. Effects of different doses of a ClO₂-generating granular mixture on Diplodia stem-end rot (*Lasiodiplodia theobromae*) in inoculated Tango mandarin at different infection stages after 24 h of ClO₂ exposure at 20°C and 2 days of incubation at 30°C and 90% RH

Treatment ^x	Incidence of Diplodia stem-end rot (%)			
	0 h ^y (test 1)	6 h (test 2)	12 h (test 3)	24 h (test 4)
Natural control	0 c	0 e	0 c	0 c
Inoculated control	95.6 a A ^z	86.7 a AB	82.7 a B	85.4 a B
ClO ₂ granular dose (g)				
1	68.6 b A	62.2 b A	66.7 b A	66.7 b A
2	51.0 b BC	42.2 c	72.0 ab AB	68.5 b AB
3	17.7 c B	28.9 d B	69.3 b A	63.0 b A

^x Natural control = noninoculated fruit without ClO₂ treatment; inoculated control = inoculated fruit without ClO₂ treatment.

^y Fruits were preincubated for 0, 6, 12, and 24 h, respectively, after inoculation and before ClO₂ exposure.

^z The lowercase letters a to d in the same column indicate significant differences among the treatments based on Duncan's multiple range test ($P = 0.05$). The capital letters A to C in the same row indicate significant differences among different preincubation times based on Duncan's multiple range test ($P = 0.05$).

Fig. 3. Mycelial growth of *Lasiodiplodia theobromae* from potato dextrose agar plugs (10 plugs per petri dish) in the absence or presence of Tango mandarins (average total weight = 2,225 ± 7.3 g/7.7-liter container). Petri dishes were treated with different ClO₂ doses for 24 h at 20°C in sealed plastic containers and were then incubated without ClO₂. Dishes outlined in green represent treatments that completely inhibited mycelial growth.

research, various factors including temperature, RH, light, and the presence of fruit can result in the loss of ClO_2 concentration in a sealed tank (Arango et al. 2016; Sun et al. 2017b). This suggests that the ClO_2 concentration loss observed in this study was mainly because of the absorption by fresh fruit, PDA medium, and even the container material. The difference in ClO_2 concentrations between the two experiments (with or without fruit) indicates that fresh citrus fruits are primarily responsible for ClO_2 absorption under the test conditions. Previous research on berries reported similar results, with ClO_2 absorption by the fruit being a rapid process in which the strawberries consumed 15% of a 5 ppm of ClO_2 treatment within 7 min (Arango et al. 2014). In another study, the ClO_2 concentration in an empty tank approached 9 ppm in 2 h, whereas the concentration in the tank containing cantaloupes was near zero (Smith et al. 2015). In our current study, the in vitro results were supported by the ClO_2 concentration change curves where the ClO_2 level of 1.5 g of granular mixture with fruit declined to less than the level of 0.5 g of granular mixture without fruit, which fell below the minimum inhibitory ClO_2 concentration for *Diplodia* stem-end rot control. Since citrus fruit absorbs ClO_2 , the actual available ClO_2 that kills or

Fig. 4. Effects of different doses of a ClO_2 -generating granular mixture on *Diplodia* stem-end rot (*Lasiodiplodia theobromae*) in naturally infected U.S. Early Pride mandarin. Fruits were treated with 0 (control), 3, 6, and 9 g of ClO_2 -generating granular mixture, at 20°C for 24 h, degreened with 5 ppm of ethylene at 30°C and 90% RH for 48 h, and then incubated at 20°C and 75% RH for up to 2 weeks. Three similar results of the repeated experiments were combined after Bartlett's test of homogeneity of variance for all the treatments. Letters above the bars indicate significant differences among treatments based on Duncan's multiple range test ($P < 0.05$).

Table 2. Effects of different doses of a ClO_2 -generating granular mixture on fruit quality of U.S. Early Pride mandarin after 24 h of ClO_2 exposure at 20°C, 48 h of ethylene degreening (5 $\mu\text{l/liter}$ of ethylene, 30°C, and 90% RH), and 2 weeks of incubation at 20°C

Treatment	Weight loss (%)	Firmness (N)	Puncture resistance (N)	TSS ^v (%)	TA ^w (%)
Initial ^x		165.5 a	15.1 a	8.7 a	0.5 a
Control ^y	5.2 a ^z	110.1 b	11.6 b	8.3 b	0.4 b
ClO_2 -generating mixture (g)					
3	5.1 a	120.7 b	12.5 b	8.3 b	0.5 a
6	4.6 a	120.8 b	14.5 a	8.3 b	0.5 a
9	5.4 a	120.3 b	15.3 a	8.4 b	0.5 a

^v TSS = total soluble solid content.

^w TA = titratable acid content.

^x Initial means fruit qualities immediately before the ClO_2 treatments.

^y Control = fruit not treated with ClO_2 .

^z Letters within a column indicate significant difference based on Duncan's multiple range test ($P = 0.05$).

suppresses the fungal pathogens should be considered. The time duration of ClO_2 releasing from its generating granular mixtures should also be considered. A fast-release type of ClO_2 -generating granular mixtures was used in this study, and it released >90% of its ClO_2 in about 30 min after the initial application (date not shown). Therefore, the determination of optimum application rates of ClO_2 should consider the decay control efficacy, controlled ClO_2 release types, fruit absorption, and the potential impact of fruit peel physiology and quality by ClO_2 .

Postharvest *Diplodia* stem-end rot results from latent *L. theobromae* infections in the field before harvest (Ismail and Zhang 2004; Zhang 2014). After harvest, the pathogen starts to further infect fruit tissues after an abscission zone forms between fruit and fruit button. Therefore, when inoculated fruit were used for evaluating the efficacy of ClO_2 for *Diplodia* stem-end rot control in this study, the fruit button tissues (calyx and disk) were removed before inoculating the fruit stem-end cavity with *L. theobromae* mycelia. Inoculated fruit were also incubated at 30°C, which is the optimal temperature for this fungal growth (Zhang 2007). Under these conditions, the pathogen readily infected and quickly spread into fruit tissues. The time between fruit inoculation and ClO_2 treatment resulted in significant differences in disease incidence and decay control efficacy (Table 1). Different incubation periods for inoculated fruit before ClO_2 treatment in this study was to simulate the relationship between *L. theobromae* natural infection process and the application time of the decay control measures. Thus, when fruit were exposed to ClO_2 immediately (0 h) after inoculation, ClO_2 was most effective because the contact occurred before the fungus could infect fruit tissues. However, after 12 and 24 h postinoculation, *L. theobromae* had become more established resulting in almost as much infection as in the untreated, inoculated control.

Although a number of studies reported good penetration ability of ClO_2 gas into the fruit stem scar (Han et al. 2001; Lee et al. 2015; Park and Kang 2015; Wang et al. 2019), our results indicated less penetration activity as it was effective only before the fungus penetrated inside fruit tissues (Table 1). However, the interactions between ClO_2 gas, fruit tissues, fruit physiologic responses, and pathogens within the tissue, especially under different environmental conditions are still not clear and additional studies are needed.

In Florida, citrus fruit harvested early in the season are often subjected to degreening treatment (using ethylene) to stimulate the breakdown of chlorophyll in the flavedo and reveal the yellow to orange carotenoid pigments before marketing the fruit. It has been reported that ethylene degreening practice significantly increases the development of *Diplodia* stem-end rot and anthracnose (caused by *C. gloeosporioides*) on citrus fruit (Ismail and Zhang 2004; Plaza et al. 2004; Zhang 2014). This is largely because of ethylene

Table 3. Effects of different doses of a ClO_2 -generating granular mixture on the pH of fruit albedo of U.S. Early Pride mandarin immediately before and after 24 h of ClO_2 exposure in 7.7-liter sealed containers

ClO_2 granular dose (g)	pH of fruit albedo		
	Initial ^v	Final ^w	
		Original ^x	Fresh ^y
0	5.9 aA ^z	5.9 aA	5.8 aA
1	6.0 aA	5.6 bC	5.8 aB
3	6.0 aA	5.4 cC	5.8 aB
6	5.9 aA	5.1 dC	5.8 aB
9	6.0 aA	4.8 eC	5.8 aB

^v Initial data were measured immediately before ClO_2 treatments.

^w Final data were measured immediately after ClO_2 treatments.

^x Original data were measured at wounds made before the ClO_2 treatments and were exposed to ClO_2 for 24 h before the final pH measurement.

^y Fresh data were measured at new wounds made after the ClO_2 treatments.

^z The lowercase letters (a to e) in the same column indicate significant differences among the treatments based on Duncan's multiple range test ($P = 0.05$). The capital letters (A to C) in the same row indicate significant differences among the initial and final values based on Duncan's multiple range test ($P = 0.05$).

promoting fruit senescence and abscission zone formation leading to greater decay (Zhang 2014). Our current results indicate that ClO₂ treatment could be more effective for *Diplodia* stem-end rot control when it is applied before or during fruit degreening.

Other decays such as green mold (*P. digitatum*) can also be a problem on fresh Florida citrus. Testing conditions in our studies (i.e., high fruit incubation temperatures, ethylene degreening and fruit inoculation) favored *L. theobromae* growth and infection. Thus, *Diplodia* stem-end rot developed rapidly, whereas other diseases were rare. Thus, ClO₂ effects on these other decays is not clear and further study is needed.

Effective control of citrus postharvest diseases requires an integrated management system including practices before and after harvest. During postharvest handling, fruit are commonly treated with fungicides such as thiabendazole (TBZ), imazalil and sodium-o-phenylphenate (SOPP) for decay control. TBZ is generally considered more effective for *Diplodia* decay control than imazalil and SOPP (Ismail and Zhang 2004). Fungicides are commonly applied to citrus fruit as an aqueous drench before degreening, aqueous treatments on the packing line, and/or added to the fruit coating to provide continued disease suppression during fruit shipping, storage, and marketing. The use of ClO₂ could provide an additional measure to be included in the current citrus decay control system to minimize fruit decay losses, maintain citrus fruit quality, and prolong fruit shelf life.

Fungicide application is the core component of the integrated postharvest decay control system of fresh citrus (Zhang 2007). However, because of increasingly restrictive regulations, environmental and health concerns, and pathogen resistance development, alternatives and safe methods to chemicals for citrus postharvest decay control are constantly needed. ClO₂ gas is a strong oxidizing agent with approximately 2.5 times higher activity than chlorine and with a broad biocidal effectiveness (Benarde et al. 1965). It has also been reported that the residues of ClO₂-treated oranges were found to be at very low levels (Trinetta et al. 2011). Therefore, the use of ClO₂ treatment as a component in a postharvest decay control system may reduce fungicide usage levels and costs, potential fungicide resistance development, and the fungicide residue problems for citrus postharvest decay control.

Fruit quality changes continuously during postharvest storage (Ladanya 2010). Both fruit weight and firmness decline after harvest, largely as a result of fruit moisture loss (Paniagua et al. 2013). However, ClO₂ treatment significantly maintained fruit puncture resistance during storage, which is similar to what was found with ClO₂-treated blueberries (Sun et al. 2014). This may be explained by the inhibitory effect of ClO₂ on enzymes such as peroxidase and polyphenol oxidase (Wang et al. 2011), respiration (Guo et al. 2014), metabolism (Gómez-López et al. 2008), and ethylene production (Aday and Caner 2011), which all play an important role in fruit softening. Reduced fruit respiration may be reflected in fewer changes in TSS and TA (Aday et al. 2013). However, no significant differences in TSS were found among the treatments and control. TA, mainly malic and citric acid, is directly related to the concentration of organic acids present in fruit and plays an important role in pH regulation of the cell (Kader 1991). In this sense, the higher TA values of ClO₂-treated fruit compared with the control suggests that ClO₂ slowed respiration and significantly maintained the organic acid content.

High concentrations of ClO₂ increased bleaching on fruit flavedo, which resulted in low brightness levels (Sun et al. 2019). Fruit color changed from vivid to gray, which indicated fruit discoloration under high concentration of ClO₂ (Sy et al. 2005). However, no significant changes in color after ClO₂ exposure were detected in the current studies. Our study also showed that fruit albedo pH significantly decreased in both original and fresh wounds after ClO₂ exposure. This is probably because of the oxidation of carbohydrates to carboxylic acid by dissolved ClO₂ in fruit tissue (Aday and Caner 2011). Additionally, the original wounds were open during the whole course of exposure and were in direct contact with ClO₂. Thus, it is believed the higher concentration led to more absorption of ClO₂, which caused the decrease in albedo pH. Conversely, albedo of fresh wounds was not directly exposed to ClO₂ and maintained

significantly higher pH than that of original wounds. Thus, fruit flavedo tissues may provide some barrier property against ClO₂ penetration.

High ClO₂ concentrations can cause phytotoxicity, physiologic defects, or injury to fruits and vegetables. Exposure to higher ClO₂ concentrations in grapefruit packaging during storage caused fruit peel browning, whereas lower rates did not (Sun et al. 2017a). Mahovic et al. (2007) reported that the higher doses of ClO₂ caused stem scars on tomato fruit to become cracked, sunken, and bleached. However, under our test conditions, we did not observe phytotoxicity injury or obvious fruit peel disorders from any of the ClO₂ treatments.

ClO₂ exists as a greenish yellow to orange gas at room temperature, and it is explosive in air when concentrations exceed 10% vol/vol (WHO 2002). Additionally, ClO₂ is harmful and a severe irritant to skin, eyes, and the respiratory tract (WHO 2002). The U.S. Occupational Safety and Health Administration (OSHA) permissible exposure limits for ClO₂ gas are as follows: 0.1 mg/liter of 8-h time-weighted average (TWA), and 0.3 mg/liter of short-term exposure limit (STEL). ClO₂ gas was generated in the current study by combining two solid granular products. Therefore, operator exposure to ClO₂ gas is limited. In addition, the fast-release type of ClO₂-generating products used released most of their ClO₂ in about 30 min, so that most ClO₂ will be absorbed by the citrus fruit in the closed atmosphere well before the rooms or containers are opened again, limiting exposure to operators. Therefore, with personal protective equipment, the safety of applying these types of ClO₂ treatments on citrus fruit should be secured.

Many reports have shown that ClO₂ gas treatments of various fruits and vegetables can reduce populations of food-borne pathogens such as *Escherichia coli* and *Salmonella* species (Lee et al. 2004; Sun et al. 2017b). ClO₂ gas at 5 mg/liter completely inhibited the growth of *E. coli* on inoculated grapefruit after 24 h of incubation at 20°C (Sun et al. 2017b). It had been reported that the residue levels of ClO₂ on ClO₂ gas-treated oranges were much lower (Trinetta et al. 2011). Fresh citrus such as mandarins are generally consumed by removing the fruit peels, which would further reduce ClO₂ residues of treated fruit if any were initially present. Our current study also showed that ClO₂ gas had no negative effects on fruit quality characteristics including weight loss, firmness, puncture resistance, titratable acids, total soluble solids and rind color. This suggests that the ClO₂ gas treatment has good potential to maintain fresh citrus fruit quality and safety for the final consumers.

In summary, the ClO₂ gas treatments reported here show promise for reducing *Diplodia* stem-end rot of citrus fruit. The antifungal activity of gaseous ClO₂ against *L. theobromae* was demonstrated in vitro and in vivo. In addition, ClO₂ treatments generally resulted in no negative effects on fruit quality and, in some cases, maintained peel puncture resistance and juice TA during storage. This suggests that ClO₂ gas delivered by this new application technology have potential for integration into an effective decay control system to prolong fruit quality and shelf life.

Literature Cited

- Aday, M. S., Buyukcan, M. B., and Caner, C. 2013. Maintaining the quality of strawberries by combined effect of aqueous chlorine dioxide with modified atmosphere packaging. *J. Food Process. Preserv.* 37:568-581.
- Aday, M. S., and Caner, C. 2011. The applications of 'active packaging and chlorine dioxide' for extended shelf life of fresh strawberries. *Packag. Technol. Sci.* 24:123-136.
- Allende, A., Tomás-Barberán, F. A., and Gil, M. I. 2006. Minimal processing for healthy traditional foods. *Trends Food Sci. Technol.* 17:513-519.
- Arango, J., Rubino, M., Auras, R., Gillett, J., Schilder, A., and Grzesiak, A. L. 2016. Evaluation of chlorine dioxide as an antimicrobial against *Botrytis cinerea* in California strawberries. *Food Packag. Shelf Life* 9:45-54.
- Arango, J., Rubino, M. I., Auras, R., Rachford, A. A., Bai, Z., Grzesiak, A. L., and Kijchavengkul, T. 2014. *In situ* quantification of chlorine dioxide gas consumption by fresh produce using UV-visible spectroscopy. *J. Food Eng.* 131:75-81.
- Barmore, C., and Brown, G. 1985. Influence of ethylene on increased susceptibility of oranges to *Diplodia natalensis*. *Plant Dis.* 69:228-230.
- Benarde, M. A., Israel, B. M., Oliveri, V. P., and Granstrom, M. L. 1965. Efficiency of chlorine dioxide as a bactericide. *Appl. Environ. Microbiol.* 13:776-780.

- Beuchat, L. R. 1996. Pathogenic microorganisms associated with fresh produce. *J. Food Prot.* 59:204-216.
- Brown, G. E. 1975. Factors affecting postharvest development of *Colletotrichum gloeosporioides* in citrus fruits. *Phytopathology* 65:404-409.
- Chen, H., McCollum, G., Baldwin, E., and Bai, J. 2016. Impacts of huanglongbing symptom severity on fruit detachment force and mechanical properties of sweet oranges (*Citrus sinensis*). *HortScience* 51:356-361.
- Couri, D., Abdel-Rahman, M. S., and Bull, R. J. 1982. Toxicological effects of chlorine dioxide, chlorite and chlorate. *Environ. Health Perspect.* 46:13-17.
- Du, J., Han, Y., and Linton, R. 2002. Inactivation by chlorine dioxide gas (ClO₂) of *Listeria monocytogenes* spotted onto different apple surfaces. *Food Microbiol.* 19:481-490.
- Gómez-López, V. M., Ragaert, P., Jeyachandran, V., Debevere, J., and Devlieghere, F. 2008. Shelf-life of minimally processed lettuce and cabbage treated with gaseous chlorine dioxide and cysteine. *Int. J. Food Microbiol.* 121:74-83.
- Guo, Q., Wu, B., Peng, X., Wang, J., Li, Q., Jin, J., and Ha, Y. 2014. Effects of chlorine dioxide treatment on respiration rate and ethylene synthesis of postharvest tomato fruit. *Postharvest Biol. Technol.* 93:9-14.
- Han, Y., Linton, R., Nielsen, S., and Nelson, P. 2001. Reduction of *Listeria monocytogenes* on green peppers (*Capsicum annuum* L.) by gaseous and aqueous chlorine dioxide and water washing and its growth at 7°C. *J. Food Prot.* 64:1730-1738.
- Ismail, M., and Zhang, J. 2004. Post-harvest citrus diseases and their control. *Outlooks Pest Manag.* 15:29-35.
- James, J. 2006. Overview of microbial hazards in fresh fruit and vegetables operations. Pages 1-36 in: *Microbial Hazard Identification in Fresh Fruit and Vegetables*. J. James, ed. John Wiley And Sons Inc., Somerset, NJ.
- Kader, A. A. 1991. Quality and its maintenance in relation to the postharvest physiology of strawberry. Pages 145-152 in: *The Strawberry Into the 21st Century*. A. Dale and J. Luby, eds. Timber Press, Portland, OR.
- Keskinen, L. A., Burke, A., and Annous, B. A. 2009. Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157: H7 from lettuce leaves. *Int. J. Food Microbiol.* 132:134-140.
- Ladanyia, M. 2010. *Citrus Fruit: Biology, Technology and Evaluation*. Academic Press, San Diego, CA.
- Lee, Y., Burgess, G., Rubino, M., and Auras, R. 2015. Reaction and diffusion of chlorine dioxide gas under dark and light conditions at different temperatures. *J. Food Eng.* 144:20-28.
- Lee, S.-Y., Costello, M., and Kang, D.-H. 2004. Efficacy of chlorine dioxide gas as a sanitizer of lettuce leaves. *J. Food Prot.* 7:1371-1376.
- Mahovic, M. J., Tenney, J. D., and Bartz, J. A. 2007. Applications of chlorine dioxide gas for control of bacterial soft rot in tomatoes. *Plant Dis.* 91:1316-1320.
- Montesinos-Herrero, C., Smilanick, J. L., Tebbets, J. S., Walse, S., and Palou, L. 2011. Control of citrus postharvest decay by ammonia gas fumigation and its influence on the efficacy of the fungicide imazalil. *Postharvest Biol. Technol.* 59:85-93.
- Paniagua, A., East, A., Hindmarsh, J., and Heyes, J. 2013. Moisture loss is the major cause of firmness change during postharvest storage of blueberry. *Postharvest Biol. Technol.* 79:13-19.
- Park, S.-H., and Kang, D.-H. 2015. Antimicrobial effect of chlorine dioxide gas against foodborne pathogens under differing conditions of relative humidity. *LWT Food Sci. Technol.* 60:186-191.
- Plaza, P., Sanbruno, A., Usall, J., Lamarca, N., Torres, R., Pons, J., and Viñas, I. 2004. Integration of curing treatments with degreening to control the main postharvest diseases of clementine mandarins. *Postharvest Biol. Technol.* 34:29-37.
- Ritenour, M. A., Zhang, J., and Dewdney, M. M. 2016. Control of stem-end rot of fresh citrus. *Citrus Industry* October 2016:12-15. https://crec.ifas.ufl.edu/media/crecifasufledu/extension/extension-publications/2016/2016_October_stem.pdf
- Saunt, J. 1990. *Citrus Varieties of the World. An Illustrated Guide*. Sinclair International Ltd., Norfolk, United Kingdom.
- Smilanick, J., Brown, G., and Eckert, J. 2006. The biology and control of post-harvest diseases. Pages 339-396 in: *Fresh Citrus Fruits*, 2nd Ed. W. F. Wardowski, W. M. Miller, D. J. Hall, and W. Grierson, eds. Florida Science Source, Inc., Longboat Key, FL.
- Smith, D., Ernst, W., and Herges, G. 2015. Chloroxyanion residues in cantaloupe and tomatoes after chlorine dioxide gas sanitation. *J. Agric. Food Chem.* 63:9640-9649.
- Sun, X., Bai, J., Ference, C., Wang, Z., Zhang, Y., Narciso, J., and Zhou, K. 2014. Antimicrobial activity of controlled-release chlorine dioxide gas on fresh blueberries. *J. Food Prot.* 77:1127-1132.
- Sun, X., Baldwin, E., and Bai, J. 2019. Applications of gaseous chlorine dioxide on postharvest handling and storage of fruits and vegetables – a review. *Food Control* 95:18-26.
- Sun, X., Baldwin, E., Ference, C., Narciso, J., Plotto, A., Ritenour, M., Harrison, K., Gangemi, D., and Bai, J. 2017a. The effect of controlled-release chlorine dioxide on the preservation of grapefruit. *HortScience* 52:122-126.
- Sun, X., Zhou, B., Luo, Y., Ference, C., Baldwin, E., Harrison, K., and Bai, J. 2017b. Effect of controlled-release chlorine dioxide on the quality and safety of cherry/grape tomatoes. *Food Control* 82:26-30.
- Sy, K. V., Murray, M. B., Harrison, M. D., and Beuchat, L. R. 2005. Evaluation of gaseous chlorine dioxide as a sanitizer for killing *Salmonella*, *Escherichia coli* O157: H7, *Listeria monocytogenes*, and yeasts and molds on fresh and fresh-cut produce. *J. Food Prot.* 68:1176-1187.
- Trinetta, V., Vaidya, N., Linton, R., and Morgan, M. 2011. Evaluation of chlorine dioxide gas residues on selected food produce. *J. Food Sci.* 76:T11-T15.
- Wang, L., Sokorai, K., Wu, V. C. H., and Fan, X. 2019. Gaseous chlorine dioxide maintained the sensory and nutritional quality of grape tomatoes and reduced populations of *Salmonella enterica* serovar Typhimurium. *Food Control* 96:299-309.
- Wang, Y., Wu, J., Ma, D., and Ding, J. 2011. Preparation of a cross-linked gelatin/bacteriorhodopsin film and its photochromic properties. *Sci. China Chem.* 54:405-409.
- WHO. 2002. Chlorine dioxide (Gas). Concise International Chemical Assessment Document 37. World Health Organization, Geneva, Switzerland.
- Zhang, J. 2007. The potential of a new fungicide fludioxonil for stem-end rot and green mold control on Florida citrus fruit. *Postharvest Biol. Technol.* 46:262-270.
- Zhang, J. 2014. *Lasiodiplodia theobromae* in citrus fruit (diplodia stem-end rot). Pages 309-335 in: *Postharvest Decay*. S. Bautista-Baños, ed. Academic Press, San Diego, CA.
- Zhao, W., Bai, J., McCollum, G., and Baldwin, E. 2015. High incidence of preharvest colonization of huanglongbing-symptomatic *Citrus sinensis* fruit by *Lasiodiplodia theobromae* (*Diplodia natalensis*) and exacerbation of postharvest fruit decay by that fungus. *Appl. Environ. Microbiol.* 81:364-372.