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Research Article

Chlorine Dioxide Treatment Modulates Ripening-Related Genes and Antioxidant System to Improve the Storability of Tomato

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Chlorine dioxide (ClO_2) is used to maintain quality and safety of fresh produce. However, ClO_2 action mechanism in fresh produce is unknown. In this study, firstly, we evaluated the efficacy of ClO_2 treatment on the quality, chilling injury, and calyx molding of tomatoes stored at two different temperatures. Then, ClO_2 effect on the expression of cell wall- and ripening-related genes and on the activity of antioxidant enzymes was investigated. Tomatoes were treated with gaseous ClO_2 for 15 min before transferring them to 13°C for 12 days and/or 4°C for 14 days, followed by 5 days at 20°C (shelf-life conditions). ClO_2 treatment marginally reduced the rate of respiration but did not affect ethylene production at 13°C and 4°C storage or at shelf-life conditions. When stored at 13°C, treatment with ClO_2 reduced the loss of firmness, with concomitant repression of *pectin esterase 1*, a cell wall-related gene. Additionally, at 13°C storage conditions, ClO_2 treatment maintained tomato quality in terms of soluble solid content, titratable acidity, and color and was associated with the downregulation of the ripening-related*ethylene response factors B3*/*C1*/*E*1 and the induction of antioxidant genes encoding *catalase* and *ascorbate peroxidase*. At 4°C storage conditions, ClO_2 at a concentration of 15 ppm not only maintained the firmness and quality of tomatoes but also inhibited pitting during shelf-life with a concomitant increase of catalase activity. Moreover, treatment with 15 ppm ClO_2 significantly reduced the calyx molding that is generally observed in fruits stored at 13°C and under shelf-life conditions. Hence, our results indicate that ClO_2 treatment effectively maintained tomato quality and inhibited calyx molding by partially regulating ripening-related genes and antioxidant systems, thereby improving the storability of postharvest tomatoes.

1. Introduction

The shelf-life of tomatoes is short owing to their fast-ripening nature. During storage and distribution, ripening progresses with a color change from green to red as well as softening and compositional changes in chemicals associated with flavor and aroma, such as organic acids, sugars, and other volatiles. Cold storage is a common practice to extend the shelf-life of fresh produce. However, tomatoes are prone to developing chilling injuries (CIs) under prolonged cold storage conditions, such as pitting, the development of sunken areas on the fruit, and increased susceptibility to rotting and decay [1]. CI symptoms usually become pronounced under market shelf conditions following cold storage, thereby reducing consumer desirability [1, 2]. Storage above 10°C is recommended to avoid CI in tomatoes; however, high storage temperatures accelerate softening and promote microbial growth especially on calyces, resulting in quality deterioration [1, 3–5]. The calyx is considered an indicator of the freshness and quality of the tomato fruit. Moreover, consumers are highly attracted to the green leaf aroma provided by these green parts. However, calyces are vulnerable to microbial spoilage (epiphytic bacteria and molds) during shipping and storage and are usually the first part of tomatoes to show fungal growth. The presence of mold on the calyx influences the marketability and shelf-life of tomatoes, even if the fruit itself is not infected

[5]. Thus, the maintenance of microbial safety and quality of tomatoes during storage and distribution requires investigation.

Chlorine dioxide (ClO_2) is a strong oxidizing gas used to disinfect fresh produce because of its antimicrobial efficacy against bacteria, fungi, and viruses [6–8]. As it is water-soluble, ClO_2 can be used in both aqueous and gaseous forms. However, gaseous ClO_2 is more effective for pathogen inactivation [8]. A comparison of the disinfection efficacy of various chemical and physical sanitizers revealed gaseous ClO_2 to be more effective in microbial inactivation than other sanitizers [9]. The antimicrobial effect of ClO_2 gas has been evaluated for a wide range of produce, such as spinach, potatoes, mung bean sprouts, lettuce, onions, cabbage, cantaloupe, and strawberries [6, 7]. The mechanism of action of ClO_2 against microbes involves the destabilization of the cell membrane, alteration of membrane permeability, and interruption of protein synthesis [8]. Furthermore, ClO_2 reacts with oxygenated compounds and proteins, resulting in the disruption of cellular metabolism [6].

Several studies have highlighted the efficacy of ClO_2 application in maintaining the quality of fresh produce. For instance, the controlled release of ClO_2 gas regulates the firmness of berries during storage [10]. ClO_2 exposure positively affects the composition of volatile compounds and free amino acids in citrus fruits, resulting in the retention of their distinct flavors [11]. ClO_2 exposure retains the titratable acidity (TA), soluble solid content (SSC), and vitamin C content in tomato and mulberry [12, 13]. In another study, the sensory properties of plums were preserved upon ClO_2 treatment [14]. However, ClO_2 application may negatively affect the quality of some fresh produce. For instance, ClO_2 treatment results in rapid color changes in spinach leaves and browning of grapefruit, cabbage, lettuce, peaches, and apples [15]. It is evident that different fresh produce may respond differently to ClO_2 application; hence, the optimization of treatment conditions for each produce of interest is necessary. The effect of ClO_2 on the respiration rate and ethylene biosynthesis plays a key role in ClO_2-derived quality maintenance in fresh produce [8]. However, the molecular mechanisms by which ClO_2 maintains the quality of fresh produce remain to be fully understood.

In this study, we optimized the ClO_2 treatment conditions and evaluated the efficacy of ClO_2 application on the quality, calyx molding, and CI of tomatoes stored at two different temperatures. To understand the mechanism of action of ClO_2, we assessed the impact of ClO_2 treatment on the gene expression profile of ripening-related and antioxidant genes and the activity of antioxidant enzymes.

2. Materials and Methods

2.1. Plant Materials and Treatments. "Kamma" tomatoes (*Solanum lycopersicum* Mill.) grown in Jungyeum, South Korea, were harvested at the pink-red stage and transported to the laboratory. In our preliminary experiments, tomatoes were treated with 5, 10, and 15 ppm ClO_2 for 15 min and/or 30 min. ClO_2 at 5 ppm did not have any effect on CI and calyx molding (Figure S1). Additionally, ClO_2 effect on CI and calyx molding was not significantly different when the treatment time was extended from 15 min to 30 min under our experimental conditions (data not shown). Hence, the fruits were treated with 10 or 15 ppm gaseous ClO_2 (mixed with ambient air) using a ClO_2 generator (CA300, South Korea) or left untreated (control) inside a commercial cardboard box for 15 min in a closed chamber. Twenty boxes containing 20 fruits each were used for each treatment. The ClO_2 concentration in the closed chamber was verified using a built-in ClO_2 meter. Following treatment, the fruit-containing boxes were covered with a plastic film and transferred to 13°C for 12 d or 4°C (cold storage) for 14 d followed by five days at 20°C (14 + 5 d; shelf-life conditions). During storage, the relative humidity was maintained at 90 ± 5%.

2.2. Gas Chromatography Analysis. The rate of respiration and ethylene production were analyzed using gas chromatography (Bruker 450-GC; Bruker Corp, Billerica, MA, USA). One milliliter of gas was sampled using a syringe from 2-L containers with four fruits from each treatment that had been sealed for 2 h. The injection and column temperatures were 110°C and 70°C, respectively. The thermal conductivity detector and flame ionization detector used for the ClO_2 and ethylene measurements were set at 150°C and 250°C, respectively. ClO_2 and ethylene measurements were obtained from three independent replicates per treatment per day.

2.3. Fruit Quality Evaluation. On the day of evaluation, fifteen fruits per treatment were randomly sampled to assess fruit quality. Progressive changes in skin color were monitored in a fixed set of fruits per treatment using a color difference meter (Minolta CR-400; Konica Minolta, Osaka, Japan) and reported based on Hunter's scale. Firmness was analyzed using a texture analyzer (TA Plus Lloyd Instruments Ltd, Fareham, Hampshire, UK) at a speed of 2 mm/s with a plunger head of 5 mm in diameter. The total SSC of the samples was analyzed using a digital refractometer (PAL-1, ATAGO CO. LTD, Tokyo, Japan) and TA, expressed in grams of citric acid per 100 g of sample juice, was determined by titrating 5 mL of juice from the fruit with 0.1 N NaOH until a pH of 8.2 was reached. This procedure was performed using an auto pH titrator (TitroLine Easy; SCHOTT Instruments GmbH, Mainz, Germany). Fruit pitting was expressed as the percentage of fruits that exhibited pitting. The final reported pitting rate was obtained from three independent replicates per treatment per day.

2.4. Calyx Molding. Development of molding on calyx was recorded after 4, 8, and 12 d in fruits stored at 13°C; after 7 and 14 d in fruits stored at 4°C, and after 3 and 5 d at 20°C. For each treatment group, data reported are from three independent replicates (three boxes with 20 fruits each) per treatment per day.

2.5. RNA Isolation and cDNA Synthesis. The tomatoes stored at 13°C were sampled on days 0, 4, and 12, whereas those stored at 4°C were sampled on days 0, 7, and 14. Subsequently, five fruits were pooled from each sample, and the pericarp tissue was used for RNA isolation using the

cetyltrimethylammonium bromide protocol [16]. First-strand cDNA was synthesized using a ReverTraAce kit (Toyobo, Japan).

2.6. Quantitative Real-Time PCR (qRT-PCR). qRT-PCR was performed as described previously by Park et al. [17] using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Amplification was performed using the iQ™ SYBR Green Supermix (Bio-Rad) with specific primers (Table S1). qRT-PCR was performed under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 55°C or 58°C for 40 s. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression levels of the housekeeping genes *actin* and *elongation factor* 1 (*EF1*). The qRT-PCR gene expression analysis was performed using three biological replicates.

2.7. Enzyme Extraction. All enzyme extraction procedures were performed at 4°C. Powdered freeze-dried fruit tissues (0.1 g) were homogenized in 3 mL of extraction buffer (100 mM potassium phosphate buffer (pH 7.5), 1% polyvinylpolypyrrolidone, 2 mM EDTA-Na, and 1 mM PMSF). The slurry was centrifuged (15,000 rpm, 4°C, 30 min) in a refrigerated centrifuge (LaboGene 2236R, Gyrozen Co, Ltd, Daejeon, Korea) and filtered. Then, the activities of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) enzymes of the clear supernatants were immediately measured, as described below.

2.8. CAT Activity. CAT activity was measured using a method previously described by Beers and Sizer [18] with some modifications. The supernatant (50 μL) was mixed with 1 mL of sodium phosphate buffer (pH 7.0) and 500 μL of H_2O_2 (100 mM). Absorbance was recorded every 30 s for 5 min. One unit of CAT activity was defined as the change in absorbance by a factor of 0.01 at 240 nm per min. CAT activity was expressed as units per minute per gram of dry weight ($U \cdot min^{-1}\ g^{-1}$).

2.9. APX Activity. APX activity was measured according to Chen and Asada [19] with modifications. The supernatant (100 μL) was mixed with 1 mL of reaction mixture (100 mM potassium phosphate buffer, 0.1 mM H_2O_2, and 0.5 mM ascorbate). The absorbance of the mixture was measured at 290 nm every 10 s for 1 min using a spectrophotometer (Epoch 2; BioTek Industries, Highland Park, USA). One unit of APX activity was defined as a decrease in absorbance by a factor of 1 per minute under the assay conditions. APX activity was expressed as units per minute per gram of dry weight ($U \cdot min^{-1}\ g^{-1}$).

2.10. SOD Activity. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), as previously described by Rao et al., [20] with some modifications. The supernatant (20 μL) was mixed with 100 μL of 100 mM potassium phosphate buffer (pH 7.5) and 100 μL of the reaction mixture (13 mM methionine, 10 μM EDTA-Na2, 2 μM riboflavin, and 120 μM NBT). The absorbance of the mixture was measured at 560 nm using a spectrophotometer (Epoch 1; BioTek Industries, Highland Park, USA). One unit of SOD activity was defined as the amount of enzyme that produced the half-maximal inhibition of NBT reduction. SOD activity was expressed as units per minute per gram of dry weight ($U\ min^{-1}\ g^{-1}$).

2.11. Statistical Analyses. Values are presented as the mean ± standard error. Differences between groups were evaluated by analysis of variance, and means were compared with Duncan's multiple range test; statistical significance was set at $P < 0.05$. Data were analyzed using SAS version 9.2 software (SAS Institute, Cary, NC, USA). For gene expression data, statistical analysis was performed using the *t*-test using Microsoft Excel v.2010 (Seattle, Washington). *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Effect of ClO_2 Treatment on Respiration Rate and Ethylene Production. Respiration is a major factor that contributes to postharvest quality losses in fruits. Treatment with ClO_2 marginally reduced the rate of respiration in tomatoes at both storage temperatures (13°C and 4°C). At 13°C, the effect of ClO_2 on the respiration rate was limited to 1 d during storage, and no difference between the treatments was observed thereafter (Figure 1(a)). However, ClO_2-treated fruits recorded a lower respiration rate throughout the duration of storage at 4°C and at subsequent shelf-life conditions (Figure 1(b)). No significant difference in respiration rate was observed between the ClO_2 treatment at concentrations 10 and 15 ppm (Figure 1). Further, ClO_2 at 10 and 15 ppm concentrations did not affect ethylene production under either storage condition (Figures 1(c) and 1(d)).

Regardless of ClO_2 treatment and storage temperature, firmness was observed to steadily decrease over time during storage. However, ClO_2 treatment significantly delayed the loss of firmness in fruits stored at 13°C, as evidenced by the consistently higher firmness in ClO_2-treated fruits than in control fruits (Table 1). In contrast, firmness was maintained at values similar to the control even upon ClO_2 treatment during cold storage at 4°C. Further, no significant difference in firmness was observed between the treatment groups under shelf-life conditions (Table 2). ClO_2 fumigation did not affect the SSC and TA of tomatoes at either storage temperature (Tables 1 and 2). Moreover, the surface color of the fruits was not altered upon ClO_2 treatment, as evidenced by consistently similar Hue values among the different treatment groups (Tables 1 and 2).

Values within a column with different letters are significantly different at $P < 0.05$, as determined by Duncan's multiple range test.

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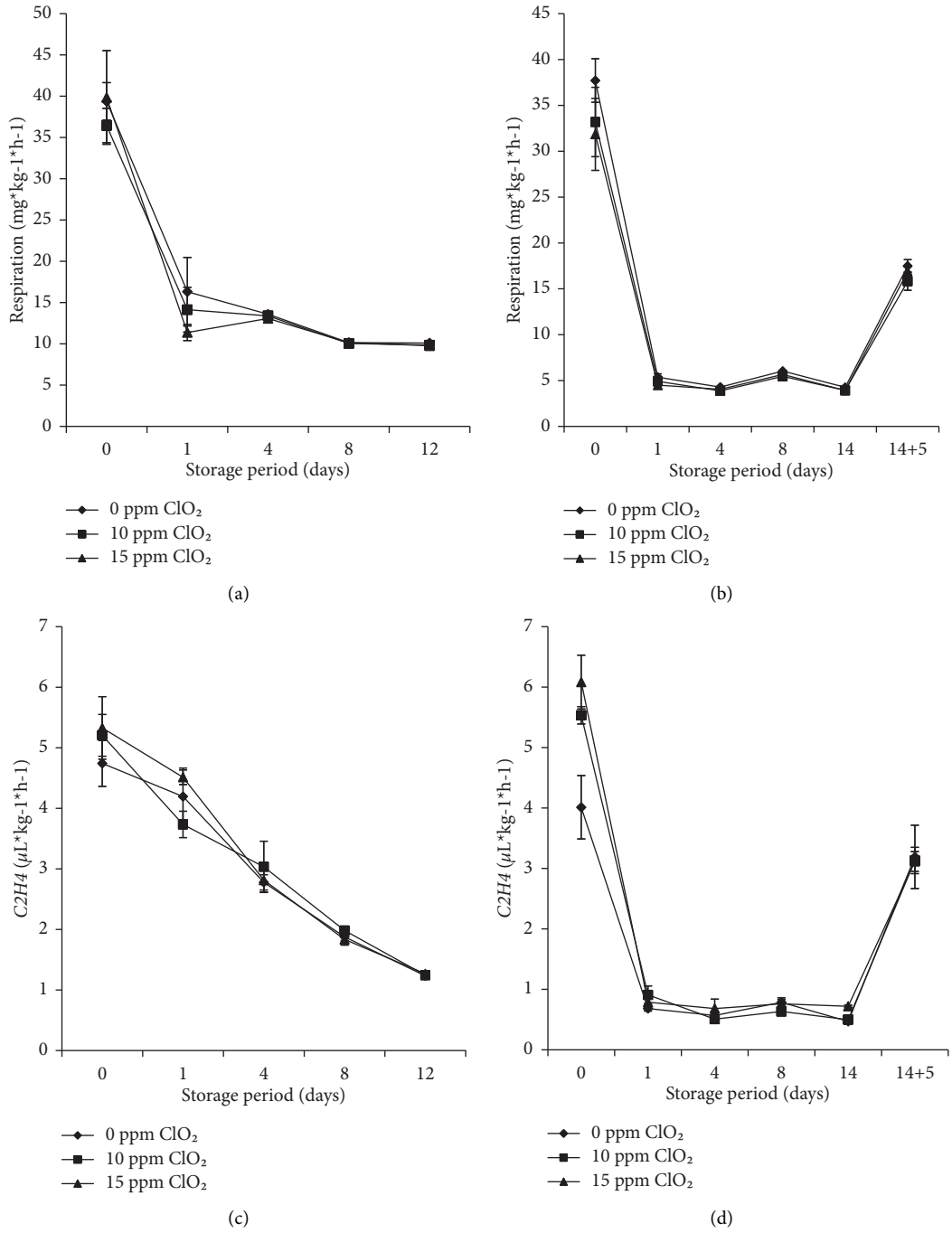


Figure 1: Effect of ClO_2 treatment on the respiration rate of tomatoes stored at (a) 13°C for 12 days and (b) 4°C for 14 days followed by 5 days at 20°C, and ethylene production in tomatoes stored at (c) 13°C for 12 days and (d) at 4 °C for 14 days followed by 5 days at 20°C. Data are represented as the mean ± standard error (SE) of three replicates. Effect of ClO_2 treatment on fruit quality.

3.2. Effects of ClO_2 Treatment Calyx Molding. No calyx molding was observed in tomatoes after 4 days at 13°C (data not shown), whereas 35% of control fruits showed calyx molding after 8 days at 13°C; this was restricted to only ~14% in the 15 ppm ClO_2 treatment group (Figure 2(b)). ClO_2 at 10 ppm showed an intermediate effect on calyx molding at 13°C (Figures 2(a) and 2(b)). No visible molding was observed on the calyces of tomatoes during cold storage (data not shown). However, upon transferring them to shelf-life conditions, molding rapidly appeared in all treatment groups. ClO_2 at 15 ppm was effective in controlling the calyx molding under shelf-life conditions (Figures 2(a) and 2(c)). On day 3 at 20°C, 15 ppm ClO_2-treated fruits demonstrated almost 50% less calyx molding than the control fruits (Figure 2(c)). However, the 10 ppm ClO_2 treatment did not significantly affect calyx molding under shelf-life conditions.

Table 1: Effect of ClO_2 treatment on quality (firmness; SSC: soluble solid content; TA: titratable acidity) and color (Hue values) in fruits stored at 13°C for 12 days.

Storage period (days)	Treatment	Firmness (N)	SSC (%)	TA (%)	Hue value
0	0 ppm ClO_2	16.73 b	4.0 a	0.58 a	63.61 a
	10 ppm ClO_2	18.70 a	4.1 a	0.59 a	63.69 a
	15 ppm ClO_2	17.60 ab	4.1 a	0.56 a	61.24 b
4	0 ppm ClO_2	13.59 b	4.0 a	0.48 a	51.94 a
	10 ppm ClO_2	15.16 a	4.1 a	0.49 a	51.56 a
	15 ppm ClO_2	14.05 ab	4.2 a	0.49 a	51.13 a
8	0 ppm ClO_2	12.34 b	4.4 a	0.53 a	47.56 a
	10 ppm ClO_2	13.42 a	4.1 b	0.47 b	47.53 a
	15 ppm ClO_2	13.59 a	4.2 ab	0.49 b	47.59 a
12	0 ppm ClO_2	11.79 a	4.1 a	0.43 a	46.66 a
	10 ppm ClO_2	12.35 a	4.2 a	0.46 a	46.42 a
	15 ppm ClO_2	11.97 a	4.2 a	0.45 a	46.62 a

Table 2: Effect of ClO_2 treatment on quality (firmness; *SSC*: soluble solid content; *TA*: titratable acidity) and color (Hue values) in tomatoes stored at 4°C for 14 days followed by 5 days at 20°C.

Storage period (days)	Treatment	Firmness (*N*)	SSC (Brix)	TA (%)	Hue value
0	0 ppm ClO_2	16.73 b	4.03 a	0.58 a	63.61 a
	10 ppm ClO_2	18.70 a	4.07 a	0.59 a	63.69 a
	15 ppm ClO_2	16.73 b	4.07 a	0.56 a	61.24 b
7	0 ppm ClO_2	15.10 a	4.13 a	0.52 a	56.90 a
	10 ppm ClO_2	15.34 a	4.13 a	0.53 a	57.87 a
	15 ppm ClO_2	15.19 a	4.10 a	0.50 a	57.39 a
14	0 ppm ClO_2	15.03 a	4.20 a	0.50 a	57.21 a
	10 ppm ClO_2	12.82 b	4.03 ab	0.50 a	58.24 a
	15 ppm ClO_2	14.53 a	3.97 b	0.54 a	57.49 a
14 + 5	0 ppm ClO_2	10.44 a	3.97 a	0.46 a	52.02 a
	10 ppm ClO_2	10.74 a	4.03 a	0.45 a	52.66 a
	15 ppm ClO_2	10.89 a	3.97 a	0.38 b	52.27 a

Furthermore, the impact of ClO_2 treatment on calyx molding diminished on the final day of storage at both temperatures (Figures 2(b) and 2(c)).

3.3. Effects of ClO_2 on CI. Prolonged exposure to cold temperatures can cause pitting, a classical symptom of CI, in tomatoes. No pitting was observed during cold storage in our experiments. However, regardless of the treatment, pitting was observed after transferring the tomatoes from cold storage to shelf-life conditions. Tomatoes treated with 15 ppm ClO_2 were less prone to pitting under shelf-life conditions than the control fruits (Figure 3). By the end of the shelf-life, more than 70% of the control fruits showed pitting, compared to only 40% of the 15 ppm ClO_2-treated fruits (Figure 3). ClO_2 at 10 ppm did not have any significant ($P < 0.05$) effect on pitting in tomatoes (Figure 3).

3.4. ClO_2-Induced Gene Expression Profiles. To elucidate the mechanisms of action of ClO_2, we examined the expression profile of cell wall–related (*pectinesterase 1 (PE1), pectin lyase (PL),* and *glucanases*), ripening-related*(ethylene response factors; ERF.B3/C1/E1*), and antioxidant genes (*APX, CAT,* and *peroxidase 42*) in fruits stored at 13°C. In addition to the higher firmness observed in ClO_2-treated fruits, the transcripts of the *PE1* gene were reduced in response to 15 ppm ClO_2 treatment on day 0; however, no difference in the expression profile between the treatment groups was observed thereafter (Figure 4(a)). Notably, the expression profiles of *glucanase* and *PL* were not altered by the ClO_2 treatment (Figure 4(a)).

We then evaluated genes associated with ripening process (*ERF.B3/C1/E1*) and found *ERF.B3* to be down-regulated upon ClO_2 treatment until day 4 at 13°C storage

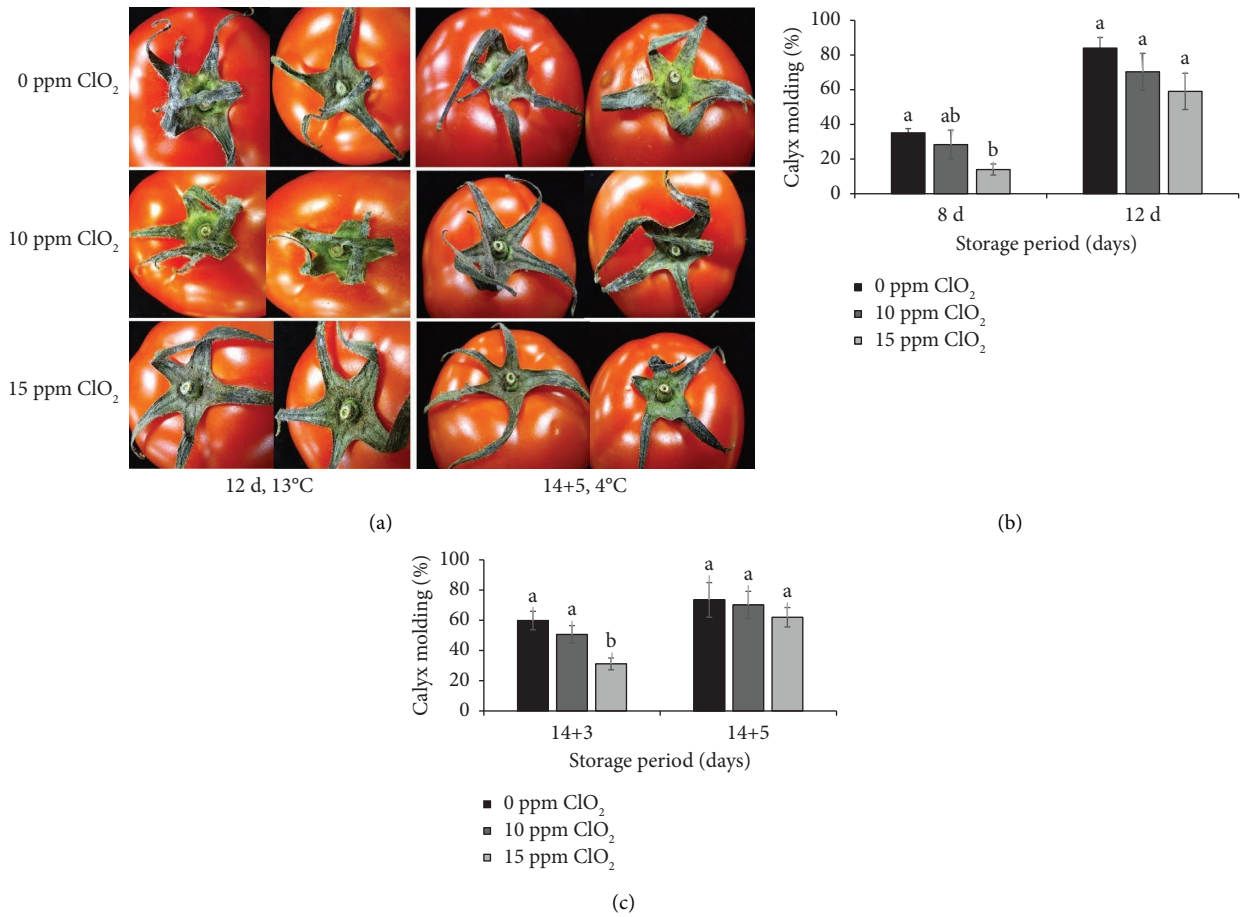


Figure 2: Effect of ClO_2 treatment on calyx molding in tomato. (a) Representative images of tomatoes with differential calyx molding after various treatments and storage conditions. (b) Calyx molding in tomatoes stored at 13°C and (c) 4°C for 14 days followed by 5 days at 20°C. Graphs plotted represent mean values ± standard error (SE) of three replicated measurements. Different lowercase letters in the graph indicate statistically significant differences at $P < 0.05$.

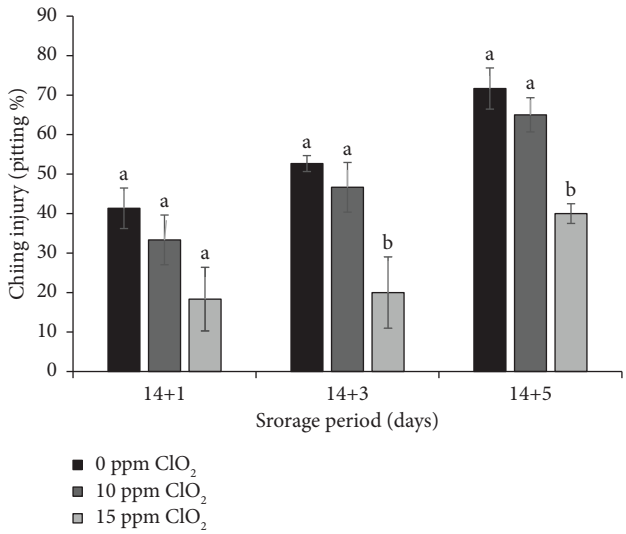
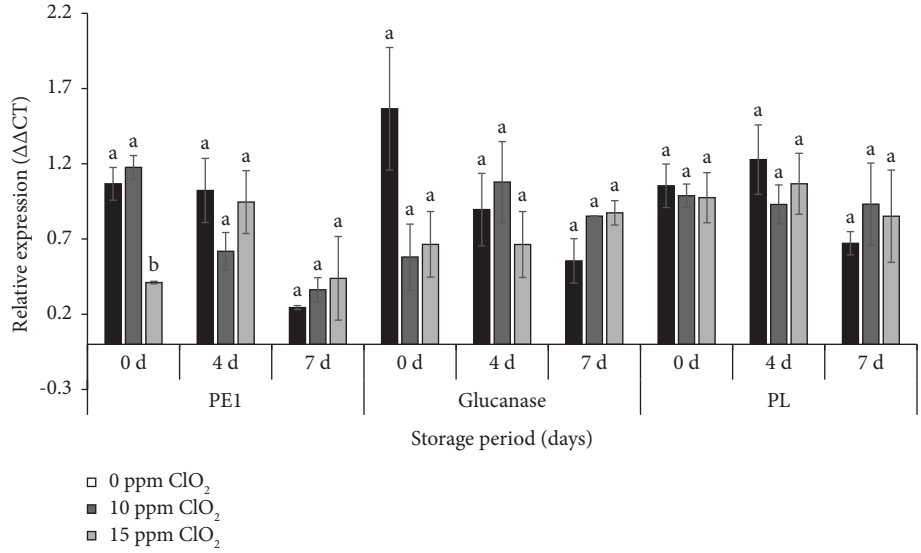


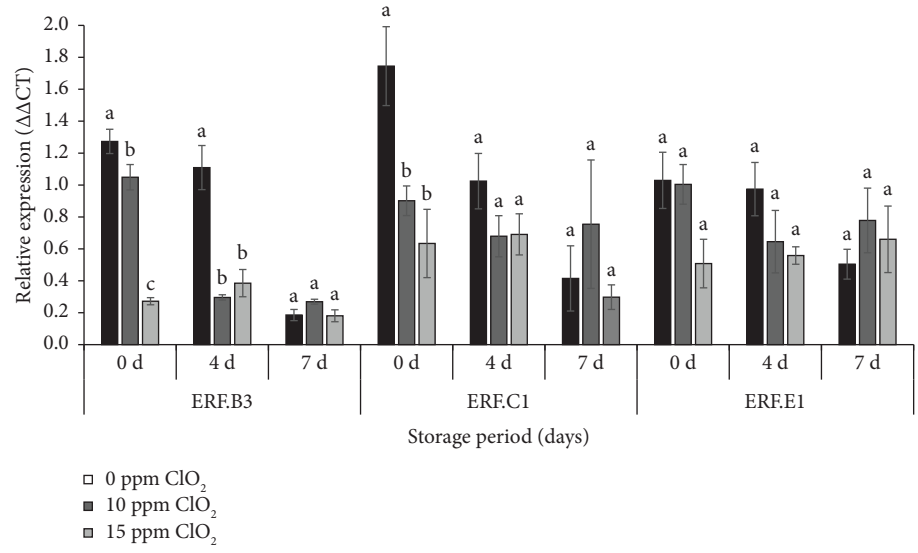
Figure 3: Effect of ClO_2 treatment on chilling injury in tomatoes stored at 4°C for 14 days followed by 5 days at 20°C. Graphs plotted represent mean values ± standard error (SE) of three replicated measurements. Different lowercase letters in the graph indicate statistically significant differences at $P < 0.05$.

conditions. Additionally, *ERF.C*1 transcripts were also suppressed in response to ClO_2 treatment on day 0 at 13°C significantly. *ERF.E1* levels were reduced by ClO_2 treatment but not statistically different (Figure 4(b)). This down-regulation of *ERF.B3* and *ERF.C*3 levels may influence ripening-associated changes in tomatoes. The expression levels of antioxidant genes *APX*, *CAT*, and *peroxidase 42* were induced in ClO_2-treated fruits when stored at 13°C. While the induction of these genes continued until day 4 at 13°C, APX was restricted to day 0. By the end of the storage period, no difference was observed in the expression levels of these genes between the treatment groups (Figure 4(c)).

Chilling temperatures destroy the balance between reactive oxygen species formation and antioxidant defense mechanisms, which causes oxidatively induced CI [1]. In our experiments, ClO_2 treatment resulted in significantly lower surface pitting (Figure 3). Hence, we examined the effect of ClO_2 treatment on the activity of the antioxidant enzymes APX, CA, and SOD during cold storage and shelf-life conditions. During 4°C storage, the activity of APX was not altered (Figure 5(a)), whereas SOD activity in 15 ppm ClO_2-treated fruits remained stable or even marginally declined, suggesting that a source of H_2O_2 not linked to SOD



(a)



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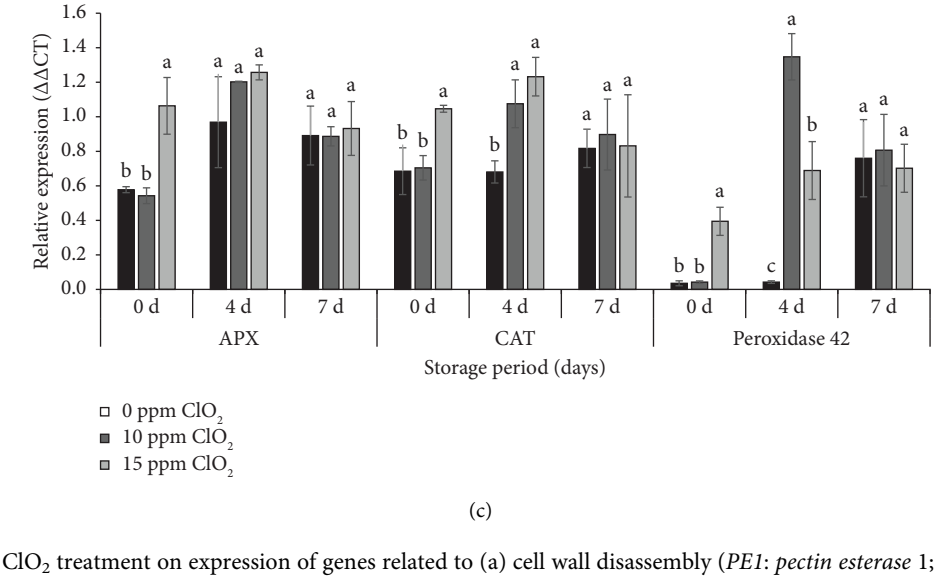

(c)

Figure 4: Effect of ClO_2 treatment on expression of genes related to (a) cell wall disassembly (*PE1*: *pectin esterase* 1; *PL*: *pectate lyase*), (b) ripening-associated ethylene response factors *(ERF.B3/C1/E1)*, and (c) antioxidant defense (*APX*: *ascorbate peroxidase*; *CAT*: *catalase; peroxidase 42*) in fruits stored at 13°C for 12 days. The bar represents the mean ± standard error of three biological replicates. Different lowercase letters in the graph indicate statistically significant differences at $P < 0.05$. ClO_2-induced antioxidant activity in tomatoes.

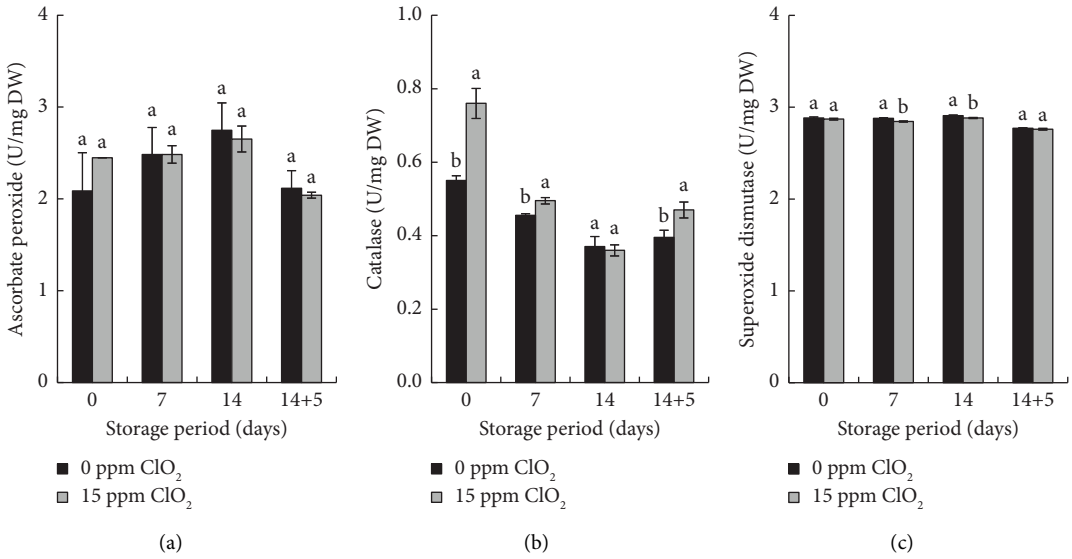

FIGURE 5: Effect of ClO_2 treatment on activity levels of antioxidant enzymes. (a) Ascorbate peroxidase (APX), (b) catalase (CAT), and (c) superoxide dismutase (SOD) in fruits stored at 4°C for 14 days followed by 5 days at 20°C. Data are shown as the mean ± standard error (SE) of three replicates. Different lowercase letters in the graph indicate statistically significant differences at $P < 0.05$.

activity may exist (Figure 5(c)). CAT activity was induced upon ClO_2 treatment during storage at 4°C (except on day 14) and shelf-life conditions, suggesting the potential involvement of this antioxidant enzyme in ClO_2-induced CI inhibition (Figure 5(c)).

4. Discussion

Respiration and ethylene biosynthesis are the key determinants of the shelf-life of tomatoes. Hence, postharvest handling methods of tomatoes primarily focus on controlling the respiratory metabolism and regulating the climacteric rise of ethylene. ClO_2 treatment is known to influence the rate of respiration and ethylene production in fresh produce [8]. For instance, gaseous ClO_2 application reduced the respiration rate in fresh-cut "Hami" melon and green peppers [21, 22]. The respiratory activity, ripeness, and senescence of peaches are controlled by ClO_2 treatment [23]. Our results corroborate these findings as treatment with ClO_2 reduced the respiration rate in tomatoes at both storage temperatures (13 and 4°C); however, the effect was more pronounced at 4°C (Figure 1). Storage of produce at low temperatures is known to inhibit respiration. Hence, the continued lower respiration rates observed here in ClO_2-treated fruits under shelf-life conditions indicate a synergetic effect of ClO_2 and cold storage (Figure 1). Guo et al. [24] reported that gaseous ClO_2 reduced ethylene biosynthesis in mature green tomatoes. However, in the present study, ethylene production was not affected. One possible reason is that the climacteric rise of ethylene in the pink-stage tomato used in this experiment has already attained before ClO_2 treatment.

Higher storage temperatures favor ripening processes that result in the loss of firmness and changes in fruit quality. We found that ClO_2-treated fruits were firmer than the control fruits throughout storage at 13°C (Table 1), indicating a positive effect of ClO_2 treatment on the regulation of firmness. Firmness was also maintained during 4°C storage (Table 2). These results are in agreement with previous reports: Islam et al. [13] reported that 12 h of gaseous ClO_2 treatment can yield tomatoes with greater firmness. Similarly, ClO_2 delayed the softening of grape tomatoes [25], and a ClO_2self-releasing sheet was effective in maintaining the firmness of cherry tomatoes [26]. ClO_2 treatment has also been shown to maintain firmness in other produce, such as strawberries, blueberries, and litchi [10, 27–29]. Interestingly, ClO_2 had no effect on firmness at 20°C, as we observed no difference in firmness between the ClO_2-treated and control groups under shelf-life conditions (Table 2). This indicates a temperature-specificity in ClO_2 action. Indeed, in a previous study, ClO_2 was more effective in maintaining blueberry firmness at 10°C than 20°C [10]. Further, the ClO_2 effect was not apparent on the firmness of strawberries at 20°C [29].

Loss of firmness in fruits is related to biochemical alterations in cell wall components and/or a loss of turgor pressure in cells due to water loss. *PE*1, *PL,* and *glucanase* are known genes involved in cell wall–associated biochemical changes during fruit ripening [30]. *PE1* is specifically expressed during the ripening process in strawberries [31]. Silencing of *PE1* in tomatoes resulted in increased levels of soluble solids and decreased levels of soluble polyuronides in cell walls, which enhanced fruit rigidity [32]. Transient genetic manipulation of *PE1* in strawberries by overexpression and silencing significantly influences the fruit firmness [33]. In this study, *PE1* transcripts were found to be reduced in response to 15 ppm ClO_2 treatment on day 0, indicating their involvement in regulating firmness (Figure 4). *PLs* are pectin-modifying enzymes that cleave glycosidic bonds via a β-elimination mechanism between

galacturonosyl residues [34, 35]. *PL* is dominant during fruit maturation, and RNA interference of *SlPL* results in enhanced fruit firmness and changes in pericarp cells [36]. Further suppression of the *PL* gene in tomatoes enhances fruit firmness and extends the shelf-life without affecting fruit quality [36, 37]. *Glucanase* is the key enzyme involved in the hydrolytic cleavage of 1,3 beta-D glucosidic linkages in beta-1,3 glucans. *Glucanase*s are expressed during the ripening and maturation of tomatoes [38]. Previously, the ripening-associated expression of *glucanase* has been shown to be cultivar-specific in three banana cultivars [39]. However, the expression of *glucanase* and *PL* was not affected by ClO_2 treatment under our experimental conditions, suggesting that *glucanase* and *PL* may not be involved in the regulation of fruit firmness by ClO_2 treatment (Figure 4).

ClO_2 treatment is known to retain color and quality in fresh produce [8]. Active packing of strawberries with ClO_2-generating sachets maintained SSC, TA, and color at 4°C [28]. Further, it has been reported that color, TA, and SSC are not significantly affected in mangoes treated with ClO_2 [40]. Similarly, we did not find any significant effect of ClO_2 treatment on SSC and TA of tomatoes. ClO_2 has previously been shown to downregulate ethylene biosynthetic pathway genes, including *ACS2, ACO1*, and *ACO3*, in mature green tomatoes and melons [22, 24]. In our experiment, the expression of *ACS2* and *ACS4* was decreased in ClO_2-treated mature green and breaker-stage tomatoes (Figure S2) but not in the pink-stage tomatoes (Figures 1(c) and 1(d)). Hence, we checked the expression of ethylene responsive genes. *ERFs* are the downstream components of ethylene signaling that mediate ethylene-dependent gene transcription and are among the largest families of plant transcription factors. *ERF.B3, ERF.C1*, and *ERF.E1* are induced during tomato ripening [41]. Suppression of *ERF.B3* has been shown to delay the onset of ripening in tomatoes [42]. In our study, *ERF.B3, ERF.C1*, and *ERF E1* levels were downregulated upon ClO_2 treatment at the beginning of storage (Figure 4). Therefore, the suppression of *ERFs* may contribute to slowing maturation processes in ClO_2-treated fruits.

The accumulation of reactive oxygen species is associated with fruit ripening and causes over ripening [43, 44]. Postharvest application of antioxidant compounds can effectively extend the shelf-life of tomatoes and other crops by reducing reactive oxygen species [45, 46]. ClO_2 treatment altered the redox status and enhanced the antioxidant capacity in longan fruit, preventing pericarp browning [47]. Chumyam et al. [48] reported that ClO_2 could restore the redox balance, leading to a reduction and delay in fruit senescence. In our experiments, ClO_2 treatment upregulated the antioxidant genes *APX, CAT,* and *peroxidase42* at 13°C storage conditions (Figure 4). An alteration in redox status in tomatoes after ClO_2 treatment may induce the expression of these antioxidant genes. The stable redox balance in ClO_2-treated tomatoes may contribute to delayed fruit maturation. Our results suggest that the ClO_2 treatment may have the potential to slowing fruit maturation via suppressing the expression of *PE1* and *ERF.B3/C1* and induction of *APX, CAT,* and *peroxidase 42* transcripts. However, further research is needed to fully discover the role of ClO_2 treatment on ripening-associated genes in tomatoes.

We verified the efficacy of ClO_2 treatment on calyx molding, considering that lower storage temperatures inhibit microbial growth. In this study, no visible mold was observed on calyces of tomatoes stored at 4°C, whereas high storage temperatures of 13°C and 20°C (shelf-life) promoted calyx molding (Figure 3). However, ClO_2 at 15 ppm effectively controlled molding on the calyces at both storage temperatures. It has been reported that ClO_2 can inactivate microbial infestation during postharvest storage [7, 8]. Bhagat et al. [49] demonstrated that treating tomatoes with 0.5 ppm ClO_2 gas for 12 min delayed the growth of natural microflora and extended its shelf-life by 7 days when stored at 22°C. Further, ClO_2 treatment significantly delayed the development of white molds and black spots in Roma tomatoes [50]. Similarly, ClO_2 treatment reduced total aerobic bacterial, yeast, and mold counts approximately by 1 log-scale in grapefruit after 6 weeks of storage at 10°C [25]. The impact of ClO_2 on microbial activity diminishes over time during storage [8]. For instance, ClO_2-generating pads effectively reduced the growth of yeast and molds for 8 days, but no effect was observed after a 12-day storage period at 2°C [40]. Similarly, the impact of ClO_2 treatment on calyx molding diminished by the end of storage at both temperatures (Figure 3).

Chilling temperatures trigger the accumulation of reactive oxygen species, which can initiate lipid peroxidation and cause oxidative damage to the cell membrane and eventual tissue deterioration [1]. Surface pitting is a visual symptom of CI in tomatoes. It is known that CI may develop during exposure to low temperatures, but the symptoms usually appear after the transfer of the produce to nonchilling temperature conditions [1]. We observed that surface pitting in tomatoes during shelf-life following cold storage was significantly inhibited by 15 ppm ClO_2 treatment (Figure 3). Plants have developed antioxidant defense mechanisms to diminish the deleterious effects of reactive oxygen species on cells. These mechanisms include antioxidant enzymes SOD, APX, CAT, peroxidases, and several nonenzymatic antioxidants. Cold stress induces the activity of APX, CAT, and SOD during cold storage and shelf-life. Interestingly, our results indicate a higher activity of CAT in 15 ppm ClO_2-treated fruits, suggesting that higher antioxidant activity after ClO_2 treatment may contribute to the inhibition of pitting in tomatoes (Figure 5). ClO_2 has been shown to increase the total antioxidant capacity of produce [51]. Wang et al. reported that ClO_2 application induces peroxidase and CAT activities in barley roots and aerial parts during seed germination. ClO_2 significantly enhances the activities of SOD, CAT, and APX in the longan pericarp [47]. Reduced CI and microbial incidence in tomato can retain greater fruit quality and improve storability.

5. Conclusions

In summary, the application of 15 ppm gaseous ClO_2 for 15 min was found to be effective in controlling the loss of firmness and maintaining the quality of tomatoes stored at 13°C and 4°C by controlling their respiratory metabolism and microbial incidence. Ripening-related*ERFs* were suppressed by ClO_2. Furthermore, the expression and activity of antioxidant genes and enzymes were modulated upon ClO_2 treatment. These findings will be useful for further understanding the molecular mechanism of action of ClO_2 for quality maintenance. Taken together, short-term 15 ppm ClO_2 treatment represents an efficient method to improve storability of tomatoes [53].

Abbreviations

APX: Ascorbate peroxidase
CAT: Catalase
CI: Chilling injury
ClO_2: Chlorine dioxide
ERF: Ethylene response factors
NBT: Nitro blue tetrazolium
PE1: Pectin esterase 1
PL: Pectin lyase
SOD: Superoxide dismutase
SSC: Soluble solid content
TA: Titratable acidity.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Table S1. Primer sequences for quantitative RT-PCR amplification. Figure S1. Effect of ClO_2 treatment on calyx molding and chilling injury in tomato. Figure S2. Effect of ClO_2 treatment on expression of ethylene biosynthetic pathway genes. (*Supplementary Materials*)

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