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## Supplementary Effect of Hydrogen Peroxide as a Pre-disinfectant for Sterilizing Rhizome Bud Explants of *Zantedeschia aethiopica* L. with Chlorine Dioxide

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*Zantedeschia* spp. (calla lily) are ornamental tuberous/rhizomatous plants of economic importance worldwide. *In vitro* micropropagation techniques have been widely used to mass-produce virus-free calla lilies. However, effective removal of contaminants is a major problem for the *in vitro* establishment of the calla lily propagation. In this study, we applied hydrogen peroxide ( $H_2O_2$ ) as a pre-disinfectant for sterilizing rhizome bud explants of *Z. aethiopica* followed by sterilization with sodium hypochlorite (NaOCl) or chlorine dioxide ( $ClO_2$ ) to eliminate *in vitro* contamination. Results showed that application of 5% (w/v)  $H_2O_2$  for 5 minutes followed by 1% (w/v) NaOCl or 60–180  $mg \cdot L^{-1}$   $ClO_2$  for 15 minutes significantly reduced the contamination rate to < 38% while maintaining the vigor of explant tissues. Surviving explants sterilized with  $H_2O_2$  followed by  $ClO_2$  exhibited a higher rate (>75%) of those showing shoot development than those sterilized with NaOCl (54%). The two-step sterilization method with  $H_2O_2$  and  $ClO_2$  synergistically optimized the disinfection efficiency and explants viability.

**Key words:** calla lily, *in vitro* contamination, pre-sterilization, shoot tip, viability

### INTRODUCTION

An optimal sterilization method for eliminating contamination from explants and for maintaining their viability is an important initial step in establishing *in vitro* cultures. Hypochlorites are the most commonly used disinfectants for sterilizing explants, generally in the forms of inorganic sodium hypochlorite (NaOCl), calcium hypochlorite, or mercuric chloride (Chen *et al.*, 2002; Maina *et al.*, 2010; Mihaljević *et al.*, 2013). Nevertheless, hypochlorites are less efficient against dormant endospores and biofilm bacteria (Rutala and Weber, 1997; Gagnon *et al.*, 2005), and only undissociated hypochlorites at neutral pH can penetrate across microbial plasma membranes, resulting in metabolic maladjustment, phospholipid destruction, and the formation of toxic chloramines (Fukuzaki, 2006).

*Zantedeschia* spp. (calla lilies), the Araceae family, are perennial bulbous plants native to southern Africa and are highly valued as cut flowers and ornamental potted plants worldwide (Ghimire *et al.*, 2012). More than 90% of *Z. aethiopica* in Taiwan is cultivated in Zhuzihu, Yang Ming Shan National Park. Calla lily festival was held annually during the blossom period of March to May with the meaning of art, culture and natural ecology, which promote the development of rural agritourism. Rhizomes and tubers have been widely utilized as explants for *in vitro* mass propagation of healthy and virus-free calla lilies (Kritzinger *et al.*, 1998; Chang *et al.*, 2003; Ebrahim, 2004). However, effective elimina-

tion of contaminants from soil-grown materials is more difficult than from non-soil-grown organs. *Z. aethiopica* is commonly cultivated in marshy areas, and the elimination of contaminants from rhizome bud explants is a puzzling challenge with *in vitro* culture. Kritzinger *et al.* (1998) reported that incubation of sterilized rhizome bud explants in antibiotics for a long period of 5 days subsequent to pretreatment with fungicides and sterilization with 2% (w/v) NaOCl decreased *in vitro* contamination rate from 90% to 10%. Other methods with 4% NaOCl for 5 minutes (Ebrahim, 2004) or pretreatment with fungicides followed by 3% NaOCl for 10 minutes (Chang *et al.*, 2003) were frequently applied for the micropropagation of *Z. aethiopica*; however, the *in vitro* contamination and survival rates were not mentioned. Nevertheless, a high concentration of hypochlorite or long exposure to fungicides or antibiotics may cause phytotoxicity and the emergence of antibiotic-resistant bacterial strains (Mngómba *et al.*, 2012).

Consequently, alternative disinfectants have been widely developed for improving the efficacy and safety of sterilization, such as hydrogen peroxide ( $H_2O_2$ ) and liquid chloride dioxide ( $ClO_2$ ), disinfectants with antibacterial, fungicidal, endosporicidal, and virucidal activities (Srebernick, 2007; Linley *et al.*, 2012).  $H_2O_2$  is a neutral and small-sized molecule that can rapidly penetrate membranes of microorganisms to trigger the production of free hydroxyl radicals and oxidation of DNA, proteins, and membrane lipids without generating toxic by-products (Linley *et al.*, 2012).  $ClO_2$  is a size-selective biocide specific to micron-sized organisms with minimal risk of developing resistant bacteria, that can rapidly penetrate biofilms in the molecular form and kill microbes living within the biofilm (Gagnon *et al.*, 2005; Herczegh *et*

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*al.*, 2013; Noszticzius *et al.*, 2013). In addition,  $\text{ClO}_2$  can also detoxify excess reactive oxygen species (ROS) and induce antioxidant defense systems to prevent membrane damage from free radicals (Chomkitichai *et al.*, 2014). For plant disease control,  $\text{ClO}_2$  exhibits inhibitory effects against phytopathogens, which cause black scurf sclerotia of potato, bacterial wilt of water convolvulus, and bacterial soft rot of calla lily (Errampalli *et al.*, 2006; Yoa *et al.*, 2010).

With advantages of high disinfection efficiency and low phytotoxicity,  $\text{H}_2\text{O}_2$  and  $\text{ClO}_2$  were used to substitute for autoclaving to sterilize culture medium of *Lilium longiflorum* (Curvetto *et al.*, 2006) and gerbera (Cardoso and da Silva, 2012), respectively. Furthermore,  $\text{H}_2\text{O}_2$  and  $\text{ClO}_2$  are also used to sterilize explants of cauliflower, yacon, and pomegranate (Bhawana *et al.*, 2015; Duan *et al.*, 2016). In this study, we evaluated the supplementary effects of  $\text{H}_2\text{O}_2$  as a pre-disinfectant followed by NaOCl and the synergistic effects of  $\text{H}_2\text{O}_2$  and  $\text{ClO}_2$  on rhizome bud explants of *Z. aethiopica* to decrease *in vitro* contamination and maintain the shoot development. The hypothesis was that  $\text{H}_2\text{O}_2$  could work synergistically with NaOCl or  $\text{ClO}_2$  to optimize the disinfection efficiency and explant viability.

## MATERIALS AND METHODS

### Plant materials

Rhizomes of *Z. aethiopica* L. 'SW 33-09' imported from California Callas (Golden State Bulb Growers, Moss Landing, CA) were purchased from Foreport Enterprises (Taipei, Taiwan). They were grown in plastic pots (18.5 cm diameter  $\times$  14.5 cm height) containing peat moss and perlite (3:1), and cultivated in a greenhouse at Chinese Culture University (121°32'21.3"E, 25°08'00.6"N) with daily irrigation. Newly formed small rhizomes on the main rhizomes (Fig. 1A) were used as the materials for *in vitro* cultures. After removing soil from the main rhizomes and thoroughly cleaning them with running tap water, small rhizomes with diameters of < 1.5 cm were used for the sterilization experiments (Fig. 1B). The surface of each small rhizome was stripped off using Kimwipes pre-soaked in household POAS detergent (Nice Co., Chiayi, Taiwan) and rinsed with running tap water. Any damaged tissues of the small rhizomes were excised with a scalpel, and the processed rhizome materials were blotted on paper towels and then air-dried for 10 minutes.

### Sterilization of rhizomes

Rhizomes were thoroughly sprayed with 75% ethanol (v/v) and allowed to stand for 45 seconds. Hydrogen peroxide (36.5% (w/v)  $\text{H}_2\text{O}_2$ , Sigma-Aldrich, St. Louis, MO), Clorox® bleach (5.25% (w/v) NaOCl, Clorox, Oakland, CA), and Debalin® (50 g·L<sup>-1</sup> aqueous  $\text{ClO}_2$  solution, Gih-Hwa Co., Kaohsiung, Taiwan) were used as sanitizer stocks and diluted with distilled-deionized water (d.d. $\text{H}_2\text{O}$ ) to the indicated concentrations.

*Experiment 1: Effect of pre-sterilization with  $\text{H}_2\text{O}_2$  followed by 1% NaOCl on explants*



**Fig. 1.** *Zantedeschia aethiopica* was used for sterilization experiments. (A) Newly formed small rhizomes on the main rhizome. (B) Small rhizomes used for sterilization experiments. Scale bar = 5 mm.

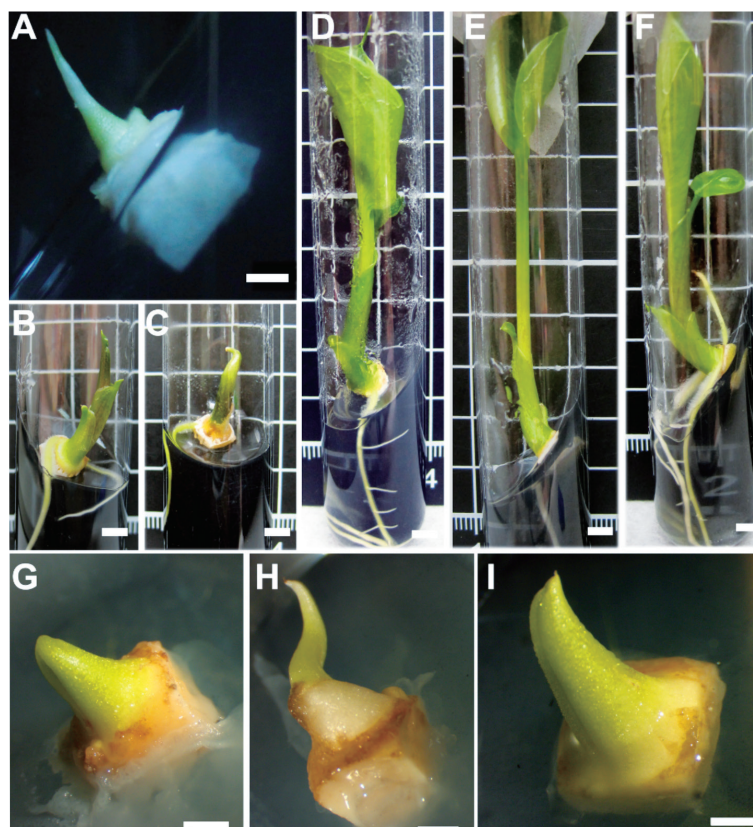
Rhizomes were pre-sterilized with 0%, 1%, 5%, or 10%  $\text{H}_2\text{O}_2$  for 5 minutes by hand agitation, rinsed three times with d.d. $\text{H}_2\text{O}$ , then sterilized with 1% NaOCl containing 0.01% (v/v) Tween-20 for 15 minutes, and rinsed three times again with d.d. $\text{H}_2\text{O}$ .

*Experiment 2: Application of 5%  $\text{H}_2\text{O}_2$  combined with  $\text{ClO}_2$  to sterilize explants*

Rhizomes were pre-sterilized with 0% or 5%  $\text{H}_2\text{O}_2$  for 5 minutes by hand agitation, rinsed three times with d.d. $\text{H}_2\text{O}$ , then sterilized with 60, 120, or 180 mg·L<sup>-1</sup>  $\text{ClO}_2$  containing 0.01% Tween-20 for 15 minutes in the dark, and rinsed three times again with d.d. $\text{H}_2\text{O}$ .

The shoot tip (1~2 mm in height) containing the basal rhizome segment was dissected from the sterilized-rhizome as the rhizome bud explant (Fig. 2A). Explants were cultured on MS basal medium containing full-strength MS salts and vitamins (Murashige and Skoog, 1962), 100 mg·L<sup>-1</sup> myo-inositol, 30 g·L<sup>-1</sup> sucrose, 1 g·L<sup>-1</sup> active charcoal, and 8 g·L<sup>-1</sup> plant agar. The pH of the medium was adjusted to 5.8, and medium was dispensed as 10-mL aliquots into test tubes (20  $\times$  150 mm). The medium at 1 kg·cm<sup>-2</sup> was autoclaved at 121°C for 15 minutes. Fourteen to 27 explants were used in each sterilization treatment with one explant per test tube, and all treatments consisted of three replicates. Explants were cultured in growth chamber (CH-202-A, Chin-Hsin, Taipei, Taiwan) at 22  $\pm$  1°C with light intensity of





**Fig. 2.** Effect of different sterilization methods on micropropagation of *Zantedeschia aethiopica* *in vitro*. (A) A rhizome bud explant sterilized with 1% NaOCl, three days of culture (Scale bar = 2 mm). (B) Shoot development in a rhizome bud explant sterilized with 5% H<sub>2</sub>O<sub>2</sub> and 1% NaOCl, two days of culture. (C) Shoot growth retardation with 10% H<sub>2</sub>O<sub>2</sub> and 1% NaOCl, five weeks of culture. Plantlet growth of a rhizome bud explant sterilized with 5% H<sub>2</sub>O<sub>2</sub> and 1% NaOCl (D), 120 mg·L<sup>-1</sup> ClO<sub>2</sub> (E), and 5% H<sub>2</sub>O<sub>2</sub> and 180 mg·L<sup>-1</sup> ClO<sub>2</sub> (F), five weeks of culture. Scale bars in B–F = 5 mm. Rhizome bud explants sterilized with 5% H<sub>2</sub>O<sub>2</sub> (G, H), and 5% H<sub>2</sub>O<sub>2</sub> and 180 mg·L<sup>-1</sup> ClO<sub>2</sub> (I), five days of culture (Scale bars = 2 mm).

50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 13-hour photoperiod. Visible contamination of the explants and shoots was recorded after *in vitro* culture for five weeks. The percentage of contamination was calculated as the number of contaminated explants divided by the total number of explants. The rate of explants showing shoot development was calculated as the number of explants showing shoot development divided by total number of surviving explants. Shoot height was the average height of shoots developing from the surviving explants.

### Statistical analysis

Data from experiments 1 and 2 were subjected to a one-way analysis of variance (ANOVA), with a significance level of  $P < 0.05$  using CoStat statistical software (Cohort Berkeley, Monterey, CA). Significant differences were determined using the Student–Newman–Keuls test. Synergistic effects of H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub> on sterilizing rhizome bud explants were analyzed by a two-factor completely randomized ANOVA, which compared H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub>. For significant values, means were separated by the least significant difference (LSD) test at  $P \leq 0.05$ , 0.01 or 0.001, using CoStat.

## RESULTS

### Effect of pre-sterilization with H<sub>2</sub>O<sub>2</sub> on the sterilization efficiency of NaOCl

Table 1 illustrates that sterilization of rhizome bud explants with 1% NaOCl for 15 minutes resulted in a high contamination percentage of 73.3% and a low survival rate of 25.9%. Pre-sterilization with both 1% and 0% (control) H<sub>2</sub>O<sub>2</sub> showed no significant effect on eliminating the contaminants *in vitro*. However, application of 5% or 10% H<sub>2</sub>O<sub>2</sub> for 5 minutes followed by 1% NaOCl significantly decreased *in vitro* contamination and increased survival rates to 60.0% and 42.9%, respectively. To evaluate the impact of H<sub>2</sub>O<sub>2</sub> on shoot growth, the shoot height and frequency of explants showing shoot development were determined, and results showed that there were no significant differences in shoot growth or development among pre-sterilization with 0% to 5% H<sub>2</sub>O<sub>2</sub>. The rates of explants showing shoot development ranged from 53.7% to 66.7%, and the average shoot height was from 6.8 to 7.7 cm after culture of five weeks, indicating that most shoot tips still retained more than 50% viability after pre-sterilization with H<sub>2</sub>O<sub>2</sub> (Fig. 2B,

**Table 1.** Supplementary effects of H<sub>2</sub>O<sub>2</sub> as a pre-disinfectant on rhizome bud explants of *Zantedeschia aethiopica* sterilized with 1% NaOCl

Concentration (%)	Contamination (%)	Survival rate (%)	Explants showing shoot development/explants surviving (%)	Shoot height (cm)
0	73.3±0.7 <sup>a</sup>	25.9±0.7 <sup>c</sup>	65.1±4.2 <sup>a</sup>	7.3±0.1 <sup>a</sup>
1	78.0±2.8 <sup>a</sup>	27.3±2.8 <sup>c</sup>	66.7±9.6 <sup>a</sup>	7.7±0.5 <sup>a</sup>
5	34.6±4.8 <sup>c</sup>	60.0±4.8 <sup>a</sup>	53.7±4.3 <sup>ab</sup>	6.8±0.1 <sup>a</sup>
10	59.9±1.9 <sup>b</sup>	42.9±1.9 <sup>b</sup>	37.9±2.8 <sup>b</sup>	1.8±0.2 <sup>b</sup>

Data were recorded five weeks after culture, calculated from three replicates each containing 15–27 samples, and expressed as the mean ± SE. Different superscript letters indicate a significant difference at  $P < 0.05$  according to a one-way ANOVA and the Student–Newman–Keuls test.

2D). However, shoot tips derived from rhizomes pre-sterilized with 10% H<sub>2</sub>O<sub>2</sub> severely lost viability, resulting in a low rate of explants showing shoot development (37.9%) and retardation of shoot growth (Fig. 2C). It should be noted that rhizomes pre-sterilized with 5% H<sub>2</sub>O<sub>2</sub> followed by 1% NaOCl effectively decreased *in vitro* contamination to less than 35% with minimal effects on shoot growth or development.

#### The synergistic effect of H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub> on sterilization

In experiment 2, each treatment was assumed to be dependent on the other. ANOVA results of main effects of H<sub>2</sub>O<sub>2</sub>, ClO<sub>2</sub>, and their interaction effect on rhizome bud explants are summarized in Table 2. Contamination, survival rates, and shoot height (with H<sub>2</sub>O<sub>2</sub> treatment) significantly differed at the levels of 0.1% or 1% for the main

effects, except for shoot height (with ClO<sub>2</sub> treatment) and shoot development which showed negligible differences. Moreover, only shoot height significantly differed in interaction effects.

To investigate the sterilization efficiency of ClO<sub>2</sub> on rhizome bud explants and its synergistic effect with 5% H<sub>2</sub>O<sub>2</sub>, six sterilization conditions, including 0% or 5% H<sub>2</sub>O<sub>2</sub> for 5 minutes in combination with 60, 120, or 180 mg·L<sup>-1</sup> ClO<sub>2</sub> for 15 minutes were implemented. Table 3 reveals that *in vitro* contamination of rhizome bud explants sterilized with 60–180 mg·L<sup>-1</sup> ClO<sub>2</sub> for 15 minutes ranged from 68.3% to 83.0%, and survival rates were only 16.7% to 31.7%. Compared to 60 mg·L<sup>-1</sup> ClO<sub>2</sub>, both 120 and 180 mg·L<sup>-1</sup> ClO<sub>2</sub> displayed better disinfection efficacies and survival rates, and shoot tips grew equally well (Fig. 2E). Notably, the rates of shoot-developing explants treated with 120 or 180 mg·L<sup>-1</sup> ClO<sub>2</sub> (75.9%–88.6%) were

**Table 2.** ANOVA of H<sub>2</sub>O<sub>2</sub>, ClO<sub>2</sub>, and their interaction for contamination, survival rate, shoot development, and shoot height of rhizome bud explants of *Zantedeschia aethiopica*

Source of variance	Degrees of freedom	Significance			
		Contamination	Survival rate	Shoot development	Shoot height
H <sub>2</sub> O <sub>2</sub>	1	***	***	ns	**
ClO <sub>2</sub>	2	**	**	ns	ns
H <sub>2</sub> O <sub>2</sub> × ClO <sub>2</sub>	2	ns	ns	ns	*

\*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ , ns: non-significant difference.

**Table 3.** The synergistic effect of H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub> on sterilization of rhizome bud explants of *Zantedeschia aethiopica*

H <sub>2</sub> O <sub>2</sub> (%)	ClO <sub>2</sub> (mg·L <sup>-1</sup> )	Contamination (%)	Survival rate (%)	Explants showing shoot development/explants surviving (%)	Shoot height (cm)
0	60	83.0±2.0 <sup>a</sup>	16.7±2.0 <sup>c</sup>	80.6±10.0 <sup>a</sup>	7.4±0.2 <sup>a</sup>
0	120	72.4±5.9 <sup>ab</sup>	27.6±5.9 <sup>bc</sup>	88.6±5.9 <sup>a</sup>	7.4±0.2 <sup>a</sup>
0	180	68.3±3.8 <sup>b</sup>	31.7±3.8 <sup>b</sup>	75.9±4.9 <sup>a</sup>	6.9±0.5 <sup>a</sup>
5	60	38.9±2.0 <sup>c</sup>	61.1±2.0 <sup>a</sup>	80.1±3.5 <sup>a</sup>	6.5±0.1 <sup>a</sup>
5	120	37.0±3.8 <sup>c</sup>	63.0±3.8 <sup>a</sup>	81.3±6.2 <sup>a</sup>	6.6±0.3 <sup>a</sup>
5	180	26.6±1.4 <sup>c</sup>	73.5±1.4 <sup>a</sup>	76.5±2.4 <sup>a</sup>	6.3±0.2 <sup>a</sup>

Data were recorded five weeks after culture, calculated from three replicates each containing 14–24 samples, and expressed as the mean ± SE. Different superscript letters indicate a significant difference at  $P < 0.05$  according to a one-way ANOVA and the Student–Newman–Keuls test.

higher than that treated with 1% NaOCl (65.1%) (Table 1). Therefore, both 120 and 180 mg·L<sup>-1</sup> ClO<sub>2</sub> could be substituted for 1% NaOCl to sterilize rhizome explants with a higher frequency of shoot development. When pre-sterilized with 5% H<sub>2</sub>O<sub>2</sub> followed by 60, 120, and 180 mg·L<sup>-1</sup> ClO<sub>2</sub>, *in vitro* contamination of rhizome bud explants was significantly decreased to low rates of 26.6% to 38.9%, and survival rates increased to high rates of 61.1% to 73.5% without any influence on shoot growth or development (Table 3), indicating that pre-sterilization with 5% H<sub>2</sub>O<sub>2</sub> can supplement the disinfection efficacy of ClO<sub>2</sub>. Moreover, two-step sterilization with H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub> improved the rate of shoot-developing explants to 76.5%–81.3%, compared to those with H<sub>2</sub>O<sub>2</sub> and NaOCl (53.7%). After pre-sterilization with H<sub>2</sub>O<sub>2</sub>, NaOCl caused more damage to rhizome bud explants than ClO<sub>2</sub>, and resulted in a lower rate of explants showing shoot development (Table 1). Furthermore, 82.6% of explant tissues (n = 23) were contaminated when sterilized with 5% H<sub>2</sub>O<sub>2</sub> only (Fig. 2G, H). Apparently, 5% H<sub>2</sub>O<sub>2</sub> and 60–180 mg·L<sup>-1</sup> ClO<sub>2</sub> have synergistic effects on eliminating contaminants for *in vitro* culture and maintaining the viability of rhizome bud explants of calla lily (Fig. 2F, 2I).

## DISCUSSION

Both H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub> act effectively against microorganisms and exhibit low phytotoxicity due to size-selectivity of ClO<sub>2</sub> and reduction of extracellular H<sub>2</sub>O<sub>2</sub> by catalases in *Lilium* (Curvetto *et al.*, 2006; Noszticzius *et al.*, 2013). They are also used as alternative sterilizers for sterilizing culture media (Cardoso and da Silva, 2012) and explants of crop (Bhawana *et al.*, 2015; Duan *et al.*, 2016). The concentration and incubation time of H<sub>2</sub>O<sub>2</sub> for *in vitro* sterilization vary and are dependent on plant species and explant type. Additionally, working concentrations of the disinfectant and sterilization time are also important for achieving the best efficiency of sterilization. A low concentration (< 0.2%) of H<sub>2</sub>O<sub>2</sub> was used to substitute for autoclaving of culture medium of *Lilium* (Curvetto *et al.*, 2006), and sterilizing orchid seeds (Snow, 1985). Miché and Balandreau (2001) sterilized rice seeds with 10% H<sub>2</sub>O<sub>2</sub> for 10 minutes followed by 1% calcium hypochlorite for 1 hour, and found that chloramines derived from the reaction of hypochlorite with ammonia caused mutations of seedlings. In this study, we combined the advantages of H<sub>2</sub>O<sub>2</sub> and ClO<sub>2</sub> in sterilizing rhizome bud explants of *Z. aethiopica* and simultaneously preventing damage to plant cells. Pre-sterilization with 5% H<sub>2</sub>O<sub>2</sub> for 5 minutes effectively enhanced the disinfection efficiency of 1% NaOCl or 60–180 mg·L<sup>-1</sup> ClO<sub>2</sub> for micropropagation of *Z. aethiopica in vitro*. Although calla lily explants sterilized with 1% NaOCl for 15 minutes exhibited a high incidence of contamination (73.3%), pre-sterilization with 5% H<sub>2</sub>O<sub>2</sub> for 5 minutes significantly compensated for the low sterilization efficiency of 1% NaOCl.

Due to chlorination-caused health risks and environment hazards, ClO<sub>2</sub> has been widely used as an alter-

native disinfectant for drinking and irrigation water instead of hypochlorite (Noszticzius *et al.*, 2013). The germicidal efficiency of ClO<sub>2</sub> (≥ 1.4 mg·L<sup>-1</sup>) is stable in a wide pH range of 3.0–9.0 and more effective than liquid chlorine in killing *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Sarcina*, and disinfection of domestic wastewater (Huang *et al.*, 1997). ClO<sub>2</sub> also killed several phytopathogens, including *Erwinia carotovora*, which causes bacterial soft rot and seriously impacts the production of calla lily (Snijder *et al.*, 2004; Yoa *et al.*, 2010). Moreover, fumigation of longan fruit with 10 mg·L<sup>-1</sup> ClO<sub>2</sub> for 10 minutes reduced the production of ROS and prevented membrane damage during storage against oxidative browning (Chomkitichai *et al.*, 2014). Cardoso and da Silva (2012) reported that shoot tips of gerbera grew and developed better in culture medium sterilized with 25–100 mg·L<sup>-1</sup> ClO<sub>2</sub> than with autoclaving. Gaseous and aqueous ClO<sub>2</sub> have also been extensively used in the surface decontamination of plant explants; however, their effective concentrations differ. Cauliflower curds were successfully sterilized with ClO<sub>2</sub> gas at high concentrations of 600 and 1500 mg·L<sup>-1</sup> for 60–360 minutes and still remained viable (Bhawana *et al.*, 2015). However, there are safety concerns with gaseous ClO<sub>2</sub> due to an explosive risk at high concentrations (Duan *et al.*, 2016). Sterilization with aqueous ClO<sub>2</sub> at low concentrations of 84–168 mg·L<sup>-1</sup> for 40–80 minutes effectively disinfected explants of low polyphenol-containing plant species, such as rice, yacon, *Pinellia ternata*, and *Isodon amethystoides* (Duan *et al.*, 2016). Nevertheless, in our study, application of 60–180 mg·L<sup>-1</sup> ClO<sub>2</sub> for sterilizing rhizome bud explants of calla lily was not efficient enough to be practical, while explants maintained higher viability than with hypochlorite. This may have been due to a short sterilization time of only 15 minutes. Compared to the combination of 5% H<sub>2</sub>O<sub>2</sub> and 1% NaOCl, pre-sterilization with 5% H<sub>2</sub>O<sub>2</sub> for 5 minutes not only supplemented the low efficiency of sterilization with 60–180 mg·L<sup>-1</sup> ClO<sub>2</sub>, but also retained a higher viability of explants for development of shoot meristems. Supposedly, ClO<sub>2</sub> contributes to the reduction reaction of H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub><sup>•-</sup> to non-toxic oxygen and triggers the antioxidant defense system to avoid oxidative damage to explants during sterilization.

## Conclusion

Our results provide an optimized strategy for sterilizing field-grown plant materials and improving the vigor and viability of explants. Two-step sterilization with 5% (w/v) H<sub>2</sub>O<sub>2</sub> and 60–180 mg·L<sup>-1</sup> ClO<sub>2</sub> offers advantages of a high sterilization efficiency and low phytotoxicity, which can overcome the disadvantages of conventional sterilization methods with hypochlorite, fungicides or antibiotics.

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