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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·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 elimination 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 (Srebernich, 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, 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, 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, H_2O_2 and 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, H_2O_2 and 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 H_2O_2 as a pre–disinfectant followed by NaOCl and the synergistic effects of H_2O_2 and ClO_2 on rhizome bud explants of *Z. aethiopica* to decrease *in vitro* contamination and maintain the shoot development. The hypothesis was that H_2O_2 could work synergistically with NaOCl or 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 × 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) H_2O_2, Sigma–Aldrich, St. Louis, MO), Clorox® bleach (5.25% (w/v) NaOCl, Clorox, Oakland, CA), and Debalin® (50 $g \cdot L^{-1}$ aqueous ClO_2 solution, Gih–Hwa Co., Kaohsiung, Taiwan) were used as sanitizer stocks and diluted with distilled–deionized water ($d.d.H_2O$) to the indicated concentrations.

Experiment 1: Effect of pre–sterilization with H_2O_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% H_2O_2 for 5 minutes by hand agitation, rinsed three times with $d.d.H_2O$, then sterilized with 1% NaOCl containing 0.01% (v/v) Tween–20 for 15 minutes, and rinsed three times again with $d.d.H_2O$.

Experiment 2: Application of 5% H_2O_2 combined with ClO_2 to sterilize explants

Rhizomes were pre–sterilized with 0% or 5% H_2O_2 for 5 minutes by hand agitation, rinsed three times with $d.d.H_2O$, then sterilized with 60, 120, or 180 $mg \cdot L^{-1}$ ClO_2 containing 0.01% Tween–20 for 15 minutes in the dark, and rinsed three times again with $d.d.H_2O$.

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 $mg \cdot L^{-1}$ myo–inoisitol, 30 $g \cdot L^{-1}$ sucrose, 1 $g \cdot L^{-1}$ active charcoal, and 8 $g \cdot L^{-1}$ plant agar. The pH of the medium was adjusted to 5.8, and medium was dispensed as 10–mL aliquots into test tubes (20 × 150 mm). The medium at 1 $kg \cdot cm^{-2}$ 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 ± 1°C with light intensity of

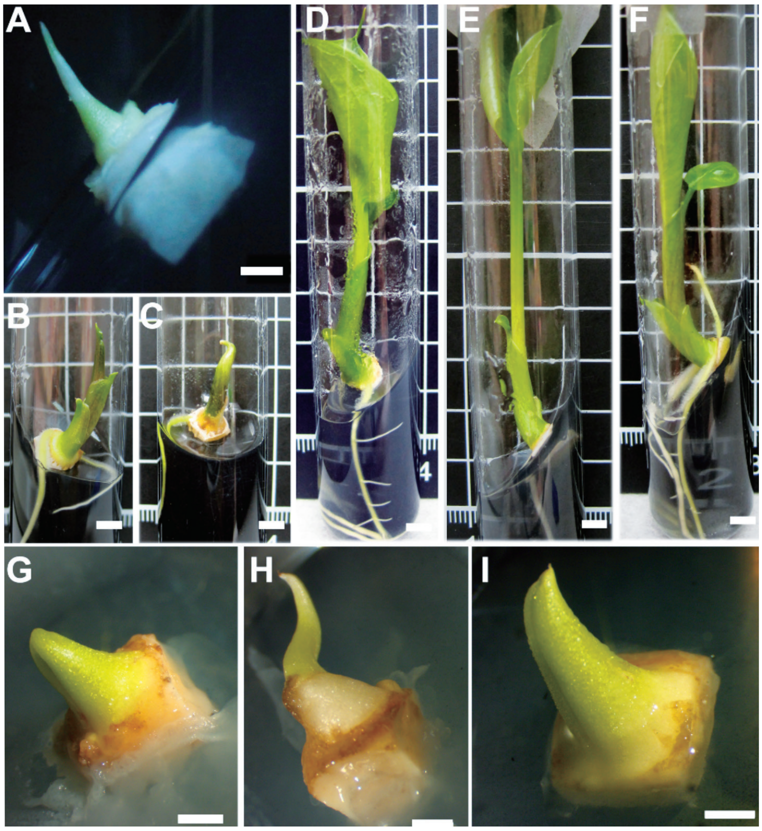

Fig. 2. Effect of different sterilization methods on micropropagation of *Zantedeschia aethiopica in vitro*. (A) A rhizome bud explant sterilized with 1% NaOCl, three days of culture (Scale bar = 2 mm). (B) Shoot development in a rhizome bud explant sterilized with 5% H_2O_2 and 1% NaOCl, two days of culture. (C) Shoot growth retardation with 10% H_2O_2 and 1% NaOCl, five weeks of culture. Plantlet growth of a rhizome bud explant sterilized with 5% H_2O_2 and 1% NaOCl (D), 120 $mg \cdot L^{-1}$ ClO_2 (E), and 5% H_2O_2 and 180 $mg \cdot L^{-1}$ ClO_2 (F), five weeks of culture. Scale bars in B–F = 5 mm. Rhizome bud explants sterilized with 5% H_2O_2 (G, H), and 5% H_2O_2 and 180 $mg \cdot L^{-1}$ ClO_2 (I), five days of culture (Scale bars = 2 mm).

50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 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_2O_2 and ClO_2 on sterilizing rhizome bud explants were analyzed by a two–factor completely randomized ANOVA, which compared H_2O_2 and ClO_2. 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_2O_2 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_2O_2 showed no significant effect on eliminating the contaminants *in vitro*. However, application of 5% or 10% H_2O_2 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_2O_2 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_2O_2. 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_2O_2 (Fig. 2B,

Table 1. Supplementary effects of H_2O_2 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^{a}	25.9±0.7^{c}	65.1±4.2^{a}	7.3±0.1^{a}
1	78.0±2.8^{a}	27.3±2.8^{c}	66.7±9.6^{a}	7.7±0.5^{a}
5	34.6±4.8^{c}	60.0±4.8^{a}	53.7±4.3^{ab}	6.8±0.1^{a}
10	59.9±1.9^{b}	42.9±1.9^{b}	37.9±2.8^{b}	1. 8±0.2^{b}

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_2O_2 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_2O_2 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_2O_2 and ClO_2 on sterilization

In experiment 2, each treatment was assumed to be dependent on the other. ANOVA results of main effects of H_2O_2, ClO_2, and their interaction effect on rhizome bud explants are summarized in Table 2. Contamination, survival rates, and shoot height (with H_2O_2 treatment) significantly differed at the levels of 0.1% or 1% for the main effects, except for shoot height (with ClO_2 treatment) and shoot development which showed negligible differences. Moreover, only shoot height significantly differed in interaction effects.

To investigate the sterilization efficiency of ClO_2 on rhizome bud explants and its synergistic effect with 5% H_2O_2, six sterilization conditions, including 0% or 5% H_2O_2 for 5 minutes in combination with 60, 120, or 180 $mg \cdot L^{-1}$ ClO_2 for 15 minutes were implemented. Table 3 reveals that *in vitro* contamination of rhizome bud explants sterilized with 60–180 $mg \cdot L^{-1}$ ClO_2 for 15 minutes ranged from 68.3% to 83.0%, and survival rates were only 16.7% to 31.7%. Compared to 60 $mg \cdot L^{-1}$ ClO_2, both 120 and 180 $mg \cdot L^{-1}$ ClO_2 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 $mg \cdot L^{-1}$ ClO_2 (75.9%–88.6%) were

Table 2. ANOVA of H_2O_2, ClO_2, 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_2O_2	1	***	***	ns	**
ClO_2	2	**	**	ns	ns
$H_2O_2 \times ClO_2$	2	ns	ns	ns	*

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

Table 3. The synergistic effect of H_2O_2 and ClO_2 on sterilization of rhizome bud explants of *Zantedeschia aethiopica*

H_2O_2 (%)	ClO_2 ($mg \cdot L^{-1}$)	Contamination (%)	Survival rate (%)	Explants showing shoot development/explants surviving (%)	Shoot height (cm)
0	60	83.0±2.0^{a}	16.7±2.0^{c}	80.6±10.0^{a}	7.4±0.2^{a}
0	120	72.4±5.9^{ab}	27.6±5.9^{bc}	88.6±5.9^{a}	7.4±0.2^{a}
0	180	68.3±3.8^{b}	31.7±3.8^{b}	75.9±4.9^{a}	6.9±0.5^{a}
5	60	38.9±2.0^{c}	61.1±2.0^{a}	80.1 ±3.5^{a}	6.5±0.1^{a}
5	120	37.0±3.8^{c}	63.0±3.8^{a}	81.3±6.2^{a}	6.6±0.3^{a}
5	180	26.6±1.4^{c}	73.5±1.4^{a}	76.5±2.4^{a}	6.3±0.2^{a}

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 $mg \cdot L^{-1}$ ClO_2 could be substituted for 1% NaOCl to sterilize rhizome explants with a higher frequency of shoot development. When pre–sterilized with 5% H_2O_2 followed by 60, 120, and 180 $mg \cdot L^{-1}$ ClO_2, *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_2O_2 can supplement the disinfection efficacy of ClO_2. Moreover, two–step sterilization with H_2O_2 and ClO_2 improved the rate of shoot–developing explants to 76.5%–81.3%, compared to those with H_2O_2 and NaOCl (53.7%). After pre–sterilization with H_{2O2}, NaOCl caused more damage to rhizome bud explants than ClO_2, 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_2O_2 only (Fig. 2G, H). Apparently, 5% H_2O_2 and 60–180 $mg \cdot L^{-1}$ ClO_2 have synergistic effects on eliminating contaminants for *in vitr*o culture and maintaining the viability of rhizome bud explants of calla lily (Fig. 2F, 2I).

DISCUSSION

Both H_2O_2 and ClO_2 act effectively against microorganisms and exhibit low phytotoxicity due to size–selectivity of ClO_2 and reduction of extracellular H_2O_2 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_2O_2 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_2O_2 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_2O_2 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_2O_2 and ClO_2 in sterilizing rhizome bud explants of *Z. aethiopica* and simultaneously preventing damage to plant cells. Pre–sterilization with 5% H_2O_2 for 5 minutes effectively enhanced the disinfection efficiency of 1% NaOCl or 60–180 $mg \cdot L^{-1}$ ClO_2 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_2O_2 for 5 minutes significantly compensated for the low sterilization efficiency of 1% NaOCl.

Due to chlorination–caused health risks and environment hazards, ClO_2 has been widely used as an alternative disinfectant for drinking and irrigation water instead of hypochlorite (Noszticzius *et al*., 2013). The germicidal efficiency of ClO_2 (≥ 1.4 $mg \cdot L^{-1}$) 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_2 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 $mg \cdot L^{-1}$ ClO_2 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 $mg \cdot L^{-1}$ ClO_2 than with autoclaving. Gaseous and aqueous ClO_2 have also been extensively used in the surface decontamination of plant explants; however, their effective concentrations differ. Cauliflower curds were successfully sterilized with ClO_2 gas at high concentrations of 600 and 1500 $mg \cdot L^{-1}$ for 60–360 minutes and still remained viable (Bhawana *et al*., 2015). However, there are safety concerns with gaseous ClO_2 due to an explosive risk at high concentrations (Duan *et al*., 2016). Sterilization with aqueous ClO_2 at low concentrations of 84–168 $mg \cdot L^{-1}$ for 40–80 minutes effectively disinfected explants of low polyphenol–containing plant species, such as rice, yacon, *Pinellia trenata*, and *Isodon amethystoides* (Duan *et al*., 2016). Nevertheless, in our study, application of 60–180 $mg \cdot L^{-1}$ ClO_2 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_2O_2 and 1% NaOCl, pre–sterilization with 5% H_2O_2 for 5 minutes not only supplemented the low efficiency of sterilization with 60–180 $mg \cdot L^{-1}$ ClO_2, but also retained a higher viability of explants for development of shoot meristems. Supposedly, ClO_2 contributes to the reduction reaction of H_2O_2 or $O_2^{\bullet -}$ 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_2O_2 and 60–180 $mg \cdot L^{-1}$ ClO_2 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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REFERENCES

Bhawana, J. M. Stubblefield, A. L. Newsome and A. B. Cahoon 2015 Surface decontamination of plant tissue explants with chlorine dioxide gas. *In Vitro Cell. Dev. Biol. Plant*, **51**: 214–219

Cardoso, J. C. and J. A. T. da Silva 2012 Micropropagation of gerbera using chlorine dioxide (ClO_2) to sterilize the culture medium. *In Vitro Cell. Dev. Biol. Plant*, **48**: 362–368

Chang, H. S., D. Chakrabarty, E. J. Hahn and K. Y. Paek 2003 Micropropagation of calla lily (*Zantedeschia albomaculata*) via *in vitro* shoot tip proliferation. *In Vitro Cell. Dev. Biol. Plant*, **39**: 129–134

Chen, L. R., J. T. Chen and W. C. Chang 2002 Efficient production of protocorm–like bodies and plant regeneration from flower stalk explants of the sympodial orchid *Epidendrum radicans*. *In Vitro Cell. Dev. Biol. Plant*, **28**: 441–445

Chomkitichai, W., A. Chumyam, P. Rachtanapun, J. Uthaibutra and K. Saengnil 2014 Reduction of reactive oxygen species production and membrane damage during storage of 'Daw' longan fruit by chlorine dioxide. *Scientia Hort.*, **170**: 143–149

Curvetto, N., P. Marinangeli and G. Mockel 2006 Hydrogen peroxide in micropropagation of *Lilium*. A comparison with a traditional methodology. *Biocell*, **30**: 497–500

Duan, Y., F. Zhao, H. Li, Y. Zhou, X. Zhu, F. Li, W. Chen and J. Xue 2016 Evaluation of aqueous chlorine dioxide for disinfecting plant explants. *In Vitro Cell. Dev. Biol. Plant*, **52**: 38–44

Ebrahim, M. K. H. 2004 Comparison, determination and optimizing the conditions required for rhizome and shoot formation, and flowering of *in vitro* cultured calla explants. *Scientia Hort.*, **101**: 305–313

Errampalli, D., R. D. Peters, K. Maclsaac, D. Darrach and P. Boswall 2006 Effect of a combination of chlorine dioxide and thiophanate–methyl pre–planting seed tuber treatment on the control of black scurf of potatos. *Crop Prot.*, **25**: 1231–1237

Fukuzaki, S. 2006 Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. *Biocontrol Sci.*, **11**: 147–157

Gagnon, G. A., J. L. Rand, K. C. O'Leary, A. C. Rygel, C. Chauret and R. C. Andrews 2005 Disinfectant efficacy of chlorite and chlorine dioxide in drinking water biofilms. *Water Res.*, **39**: 1809–1817

Ghimire, B. K., C. Y. Yu, H. J. Kim and I. M. Chung 2012 Karyotype and nucleic acid content in *Zantedeschia aethiopica* Spr. and *Zantedeschia elliottiana* Engl. *Afr. J. Biotechnol.*, **11**: 11604–11609

Herczegh, A., A. Ghidan, D. Friedreich, M. Gyurkovics, Z. Bendö and Z. Lohinai 2013 Effectiveness of a high purity chlorine dioxide solution in eliminating intracanal *Enterococcus faecalis* biofilm. *Acta Microbiol. Immunol. Hung.*, **60**: 63–75

Huang, J., L. Wang, N. Ren, F. Ma and Juli 1997 Disinfection effect of chlorine dioxide on bacteria in water. *Water Res.*, **31**: 607–613

Kritzinger, E. M., R. J. V. Vuuren, B. Woodward, I. H. Rong, M. H. Spreeth and M. M. Slabbert 1998 Elimimation of external and internal contaminants in rhizomes of *Zantedeschia aethiopica* with commercial fungicides and antibiotics. *Plant Cell Tiss. Org. Cult.*, **52**: 61–65

Linley, E., S. P. Denyer, G. McDonnell, C. Simons and J. Y. Maillard 2012 Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *J. Antimicrob. Chemother.*, **67**: 1589–1596

Maina, S. M., Q. Emongor, K. K. Sharma, S. T. Gichuki, M. Gathaara, S. M. de Villiers 2010 Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogea* L.) varieties adapted to eastern and Southern Africa. *Afr. J. Biotechnol.*, **9**: 2866–2871

Miché, L. and J. Balandreau 2001 Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Appl. Environ. *Microbiol.*, **67**: 3046–3052

Mihaljević, I., K. Dugalić, V. Tomaš, M. Viljevac, A. Pranjić, Z. Čmelik, B. Puškar and Z. Jurkovć 2013 *In vitro* sterilization procedures for micropropagation of 'Oblačinska' sour cherry. *J. Agric. Sci.*, **58**: 117–126

Mngómba, S. A., G. Sileshi, E. S. du Toit and F. K. Akinnifesi 2012 Efficacy and utilization of fungicides and other antibiotics for aseptic plant cultures. *In* "Fungicides for Plant and animal diseases", ed. by D. Dhanasekaran, InTech, Inc. Rijeka, Croatia, pp. 245–254

Murashige, T. and F. Skoog 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, **15**: 473–497

Noszticzius, Z., M. Wittmann, K. Kály–Kullai, Z. Beregvári, I. Kiss, L. Rosivall and J. Szegedi 2013 Chlorine dioxide is a size–selective antimicrobial agent. *PLoS ONE*, **8**: e79157

Rutala, W. A. and D. J. Weber 1997 Uses of inorganic hypochlorite (Bleach) in health–care facilities. *Clin. Microbiol. Rev.*, **10**: 597–610

Snijder, R. C., H. R. Cho, M. M. W. B. Hendriks, P. Lindhout and J. M. van Tuyl 2004 Genetic variation in *Zantedeschia* spp. (*Araceae*) for resistance to soft rot caused by Erwinia carotovora subsp. *carotovora*. *Euphytica*, **135**: 119–128

Snow, R. 1985 Improvements in methods for the germination of orchid seeds. *Am. Orch. Soc. Bull.*, **54**: 178–181

Srebernich, S. M. 2007 Using chlorine dioxide and peracetic acid as substitutes for sodium hypocloride in the sanitization of minimally processed green seasoning. *Ciênc. Tecnol. Aliment.*, **27**: 744–750

Yao, K. S., Y. H. Hsieh, Y. J. Chang, C. Y. Chang, T. C. Cheng and H. L. Liao 2010 Inactivation effect of chlorine dioxide on phytopathogenic bacteria in irrigation water. *J. Environ. Eng. Manage.*, **20**: 157–160