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Effect of Chlorine Dioxide (ClO_2) on Culture Medium Sterilization on Micropropagation of Persian Violet (*Exacum affine* Balf.f. ex Regel)

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Abstract The effect of chlorine dioxide (ClO_2) on culture medium sterilization in persian violet (*Exacum affine* Balf.f. ex Regel) has been studied. The culture medium was sterilized by ClO_2, which promoted shoot formation. Culture medium supplemented with 5 ppm ClO_2 gave the highest shoot at 9.27±4.13 shoot/explants. The explants were cultured on medium supplemented with 10 ppm ClO_2 which induced the highest root formation at 62.50 % and 3.61±1.00 root/explants significant different with other treatments. In addition to flower induction, above treatment gave the highest flowering at 52.38 % and blooming flower at 14.28 %. In case of concentration of culture medium, ½ MS supplemented with 10 ppm ClO_2 could be root induction at 100 % gave the highest number of root at 12.33±3.84 root/explants significant different with another treatment. The effect of agar concentration in medium, 0.60 % agar gave the best root induction at 94.44 % while 0.75% was induced flower at 52.38 % and blooming flower at 14.28 %. Another study is that effect of ClO_2 when combination with silver nitrate ($AgNO_3$) in micropropagation. The optimum concentration of $AgNO_3$ to promoted root induction and flowering was 1.0 mg L^{-1} at 33.33% root induction, 58.33 % flowering, and 50.00 % blooming flower.

Keywords: Persian violet, chlorine dioxide, sterilization, root induction, flowering

Introduction

Persian violet (*Exacum affine* Balf.f. ex Regel) which belongs to the family *Gentianaceae*, is a widely used indoor or outdoor small potted plant. Persian violet is traditionally propagated by seed and produces fragrant, blue, purple or white coloured flowers (Kapchina-Toteva *et al.*, 2005), but reduced fertility in cultivars with composed flowers has increased to need for efficient

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methods of vegetative propagation (Ørnstrup *et al*., 1993). Normally, *in vitro E. affine* Balf culture use flower buds, peduncles (Ørnstrup *et al*., 1993) and internodal sections (Ballal, 1990) as explants. Many keys are factor affecting successful on tissue culture. Ethylene is important factor in tissue culture and it play both positive and negative role in callus induction, somatic embryogenesis, organogenesis and plant regeneration (Kumar *et al*., 2009). Ethylene inhibited plant regeneration of elite wheat (Yu *et al*., 2008). $AgNO_3$ is one of inhibitor ethylene action (Beyer, 1976) because silver ions might play role in inhibitory effect in ethylene (Fei *et al*, 2000). Root induction is a finally protocol of tissue culture so root induction is important for survival after transfer to field condition. Genotype and culture medium were also reported to promote root induction (Sanputawong and Te-chato, 2008; Thawaro and Te-chato, 2010; Sirisom and Te-chato, 2014). Gelling agents in culture medium enhanced effect on root formation (Mohan *et al*., 2005). However, concentration of gelling agents affected to root induction (Suthar *et al*., 2011).

Normally, eliminate contamination microorganisms from the culture medium is using autoclave at 121 ^{o}C and pressure at 1 kg cm^{-2}) for 15 to 30 min (Torres *et al*., 1998). In case of surface explants sterilize before culture, which use mercuric chlorine (Kannan *et al.,* 2007), sodium hypochlorite (Sivanesan *et al*., 2011), cetrimide (Gantait *et al*., 2011), and dichloroisocyanuric acid Na_2 salt (Šušek *et al.,* 2002). Chlorine dioxide (ClO_2) is a potent antiprotozoan agent (Peeters *et al*., 1989), antibacterial agent (Taylor *et al.,* 2000) fungicide (Wilson *et al*., 2005), and virucide (Taylor and Butler, 1982). Bacteria may be killed effectively by CIO_2 relatively wider range of pH value (pH 3.0-8.0) (Huang *et al*., 1997). Chlorine ions are generally toxic to plants at relatively low concentrations and may cause irreversible damage to plant development (Carrillo *et al*., 1996). On the other hand, the benefits of chlorine dioxide in agriculture, which are disinfection of *Escherichia coli* and *Salmonella* sp. on agricultural product surfaces (Han *et al.,* 2000; López-Velasco *et al.,* 2012), postharvest storage quality of plum fruit (*Prunus salicina* L.) (Chen and Zhu, 2011) and sterilization in the *in vitro* culture medium (Cardoso *et al*., 2012)

Thus, the aim of this study was to evaluate the effects of ClO_2 for chemical sterilization of *in vitro* culture medium and on the development of persian violet plantlets at shoot induction, root induction, and flowering and to compare the growth of these plantlets with plantlets developed using media conventionally sterilized by autoclaving.

Materials and methods

The explants of persian violet (*E. affine* Balf.f. ex Regel) were standard medium [Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3.0 % (w/v) sucrose, 0.5 mg L^{-1} BA and 7.5 g l^{-1} agar, pH 5.8] and subcultured every 1 month. Growth conditions were 28±2°C, under 14 hours of light (23 umol$m^{-2}s^{-1}$ photosynthetic photon flux density).

Effect of chlorine dioxide on shoot induction

The explants were cultured on MS supplemented with 3% sucrose and 0.5 mg L^{-1} BA and 0.75 % agar, which sterilized by autoclaving (control) and ClO_2 at various concentration (5, 10 and 15 ppm). After 1 month of culture, the percentage of contamination medium and shoot formation, number of shoot/explants and shoot length were recorded. The data were compared with autoclaving and ClO_2 as a sterilizing agent in culture media.

Effect of chlorine dioxide on root and flower induction

The explants were cultured on MS supplemented with 3% sucrose, 0.75 % agar and without BA, which sterilized by autoclaving (control) and various concentration of ClO_2 (5, 10 and 15 ppm). After 1 month of culture, the percentage of contamination medium and root induction, number of root/explants and root length, were recorded. In parameter flower data were collected after 2 month of culture. The data were compared with autoclaving and ClO_2 as a sterilizing agent in culture media.

Effect of concentration agar on culture medium (sterilized by 10 ppm ClO_2) on root and flower induction

The explants were cultured on MS medium supplemented with 3% sucrose, various concentration of agar (0.60, 0.65, 0.70 and 0.75%) and sterilized by 10 ppm ClO_2. After culture, the day of rooted, the percentage of contamination medium, root induction, and number of root/explants and root length were recorded 1 month after incubator and flowering and blooming flower were calculated 2 months after culture.

Effect of concentration of culture medium on root induction

The explants were cultured on different concentration of MS medium (half and full strength concentration), which supplemented with 3% sucrose,

0.75% agar and without BA. All culture medium sterilized by 10 ppm ClO_2. After culture, the day of rooted, the percentage of contamination medium and root induction, and number of root/explants and root length were recorded. The data were compared with autoclaving and ClO_2 as a sterilizing agent in culture media.

Effect of $AgNO_3$ concentration on culture medium on root and flower induction

The explants were cultured on MS medium supplemented with 3% sucrose, various concentration of $AgNO_3$ (0, 0.5, 1.0 and 1.5%) solidify with 0.70 % agar and sterilized by 10 ppm ClO_2. After culture, the percentage of contamination medium, root induction, and number of root/explants and root length were recorded 1 month after incubator and flowering and blooming flower were calculated 2 months after culture.

Statistical analysis

All experiments were performed in a completely randomized design (CRD). Each consisted of four replicates per treatment and six explants were performed in each replication. Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using least significant difference (LSD) with 5 or 1% probability.

Results

Effect of chlorine dioxide on shoot induction

All explants were shoot developed on MS supplemented with 3% sucrose and 0.5 mg L^{-1} BA (0.75% agar) which steriled by autoclaving and ClO_2 after culture for 1 month. In case of medium sterilized by ClO_2, medium were not contamination. Shoot could be developed in 5 ppm ClO_2 at 9.27 ± 4.13 shoot/explants. Shoot length was the highest respond on 15 ppm ClO_2 at 7.24 ± 3.77 cm (Table 1).

Effect of chlorine dioxide on root and flower induction

All explants were root developed on MS supplemented with 3% sucrose, 0.75% agar, and sterilized by autoclaving and ClO_2 after culture for 1 month. In case of medium sterilized by ClO_2, medium were not contamination. Root could be developed in 10 ppm ClO_2, which root induction at 62.50% and

3.61±1.00 root/explant. In addition, this concentration gave the highest percentage of flowering and blooming flower at 52.38 and 14.28 %, while 5 ppm ClO_2 gave the highest root length at 7.62±2.44 cm (Table 2).

Effect of concentration of culture medium on root induction

All explants were root response on 1/2MS supplemented with 3% sucrose, 0.70% agar, and sterilized by 10 ppm ClO_2 after culture for 6 days. The best results in percentage root induction at 100%, 12.33±3.84 root/explant, and root length at 19.67±5.10 cm (Table 3) (Figure 1b)

Effect of concentration of agar on root and flower induction

After 1 month of culture, all culture medium cannot contamination. The explants were root response on MS supplemented with 3% sucrose, 0.65% agar, and sterilized by 10 ppm ClO_2. This culture medium gave the highest root induction at 83.33 %, 6.39±2.23 root/explants, and root length at 7.47±2.01 cm. The term of flowering and blooming flower, explants respond on 0.75 % agar at 52.38 % and 14.28 % (Table 4) (Figure 1c, d and e).

Effect of $AgNO_3$ concentration on culture medium on root and flower induction

After 1 month of culture, all culture medium cannot contamination. The explants were root induction respond on MS medium supplemented with 3% sucrose, 0.70% agar, 0.5 mg L^{-1} $AgNO_3$ and sterilized by 10 ppm ClO_2 at 50.00%. While 1.5 mg L^{-1} $AgNO_3$ gave the highest number of root/explant at 4.21±2.25 and root length at 10.08±5.16 cm. The term of flowering and blooming flower, explants respond on 1.0 mg L^{-1} $AgNO_3$ at 58.33% and 50.00% (Table 5) (Figure 1g).

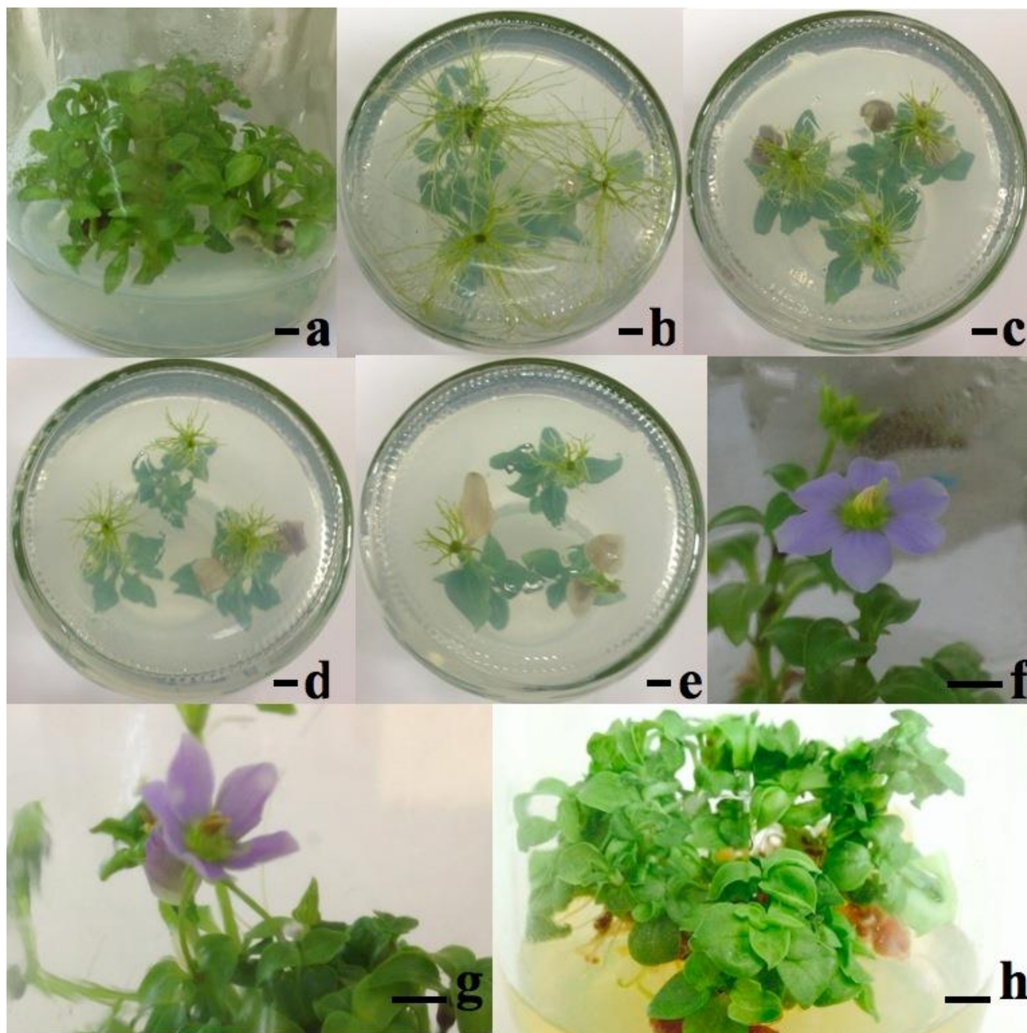

Figure 1. *In vitro* propagation of persian violet (*Exacum affine* Balf.f. ex Regel) culture on medium sterilized with 10 ppm ClO_2 (bar : 5 mm).
(a) Shoot induction on MS medium with 0.5 BA 0.75% agar. (b) Root formation on PGRs-free ½MS. Root were induced on PGRs-free MS medium supplemented with 3 different concentration of agar [(c) 0.60%, (d) 0.65% and (e) 0.70% agar]. Blooming flower of complete plantlet culture on PGRs-free MS medium with (f) 0.70% agar and (g) 0.70% agar and 1 mg L^{-1} $AgNO_3$ (h) Complete plantlets developed on PGRs-free MS supplemented with 0.70% agar and 1 mg L^{-1} $AgNO_3$.

Table 1. Effect of different concentration of chlorine dioxide on medium sterilization and shoot formation in persian violet cultured on MS medium supplemented with 3% sucrose and 0.5 mg L^{-1} BA and 0.75% agar after 1 month of culture. x

TRT	Contamination (%)	Shoot formation (%)	No. of shoot/explant	Shoot length (cm)
Autoclave	0	100	10.67±4.42	9.60±4.08^{a}
5 ppm ClO_2	0	100	9.27±4.13	5.86±3.03^{b}
10 ppm ClO_2	0	100	9.07±4.67	3.88±2.01^{b}
15 ppm ClO_2	0	100	8.40±3.93	7.24±3.77^{ab}
F test			ns	**
C.V.(%)			23.15	28.89

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$)

Table 2. Effect of different concentration of chlorine dioxide on medium sterilization and root induction in persian violet cultured on PGRs-free MS medium supplemented with 3% sucrose and 0.75% agar after 1-2 month of culture.

TRT	Contamination (%)	Root induction (%)	No. of root/explant	Root length (cm)	Flowering (%)	Blooming flower (%)
Autoclave	0	79.17^{a}	2.60±0.93	3.92±1.60	42.86	0
5 ppm ClO_2	0	33.33^{b}	3.29±0.58	7.62±2.44	28.57	0
10 ppm ClO_2	0	62.50^{b}	3.61±1.00	4.93±2.77	52.38	14.28
15 ppm ClO_2	0	58.33^{b}	2.89±0.81	5.29±3.82	28.57	0
F test		*	ns	ns		
C.V.(%)		65.02	68.86	90.76		

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$)

Table 3. Effect of concentration of culture medium on root induction in persian violet cultured on PGRs-free medium supplemented with 3% sucrose and 0.75% agar and sterilized with 10 ppm ClO_2 after 1 month of culture.

TRT	Contamination (%)	Day of rooted	Root induction (%)	No. of root/explant	Root length (cm)
½MS	0	6	100	12.33±3.84[a]	19.67±5.10[a]
MS	0	8	83.33	5.42±1.99[b]	5.37±1.93[b]
F test			ns	**	**
C.V.(%)			21.51	34.24	30.80

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$)

Table 4. Effect of four different concentration of agar on root and flower induction in persian violet cultured on PGRs-free MS medium supplemented with 3% sucrose and 0.75% agar after 1-2 month of culture.

Agar (%)	Contamination (%)	Root induction (%)	No. of root/explant	Root length (cm)	Flowering (%)	Blooming flower (%)
0.60	0	94.44	7.22±3.00[a]	8.23±3.25[a]	44.44	0.00
0.65	0	83.33	6.39±2.23[ab]	7.47±2.01[a]	11.11	0.00
0.70	0	83.33	5.42±1.99[ab]	5.37±1.93[ab]	22.22	8.33
0.75	0	66.67	3.06±1.79[b]	4.01±3.38[b]	52.38	14.28
F test		ns	**	*		
C.V.(%)		33.83	41.66	43.53		

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$)

Table 5. Effect of different concentration of $AgNO_3$ on root and flower induction in persian violet cultured on PGRs-free MS medium supplemented with 3% sucrose and 0.75% agar.

$AgNO_3$ (mgL^{-1})	Contamination (%)	Root induction (%)	No. of root/explant	Root length (cm)	Flowering (%)	Blooming flower (%)
0	0	83.33^{a}	5.42±1.99	5.37±1.93^{ab}	22.22	8.33
0.5	0	50.00^{ab}	3.96±2.03	3.67±1.96^{b}	33.33	25.00
1.0	0	33.33^{b}	4.00±0.82	4.08±2.18^{b}	58.33	50.00
1.5	0	38.89^{ab}	4.21±2.25	10.08±5.16^{a}	25.00	16.67
F test		*	ns	*		
C.V.(%)		73.72	60.54	52.04		

Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$)

Discussion

Shoot formation, in micropropagation of persian violet, ClO_2 touched on shoot induction so, medium sterilized by autoclaving promoted shoot formation then media sterilized with ClO_2. Although ClO_2 increased shoot height, fresh weight and number of leave in gerbera. In case of persian violet, ClO_2 is a few toxic to plant tissue, Srichuay and Te-chato (2014) reported that pineapples were break to growing when they were cultured in medium with high concentration of ClO_2. Effect of ClO_2 on persian violet resulted in sheath blight leaves and shoots were stop developed (Figure don't showed). *In vitro* rooting and flowering are important that plant can adaptation to acclimatization in condition for *in vitro* flowering increased the value of the plant (Yenchon and Te-chato, 2014) and assisted *in vitro* breeding programs (Pratheesh and Kumar, 2012).

Concentration of culture medium on root induction, ½ MS medium promoted a better rooting induction when compared with MS medium. In case of brahmi (*Bacopa monnieri*) half strength MS medium gave the better result in root formation than full strength (Ceasar *et al*., 2010). ½ MS medium was the best condition for rooted in persian violet. Many researcher reported that many plant species have been induced root formation in half strength MS medium as *Tuberaria major* (Gonçalves *et al*., 2010), spearmint (Fadel *et al*., 2010).

Effect of agar on root and flower induction, agar is substance for plant was stood in *in vitro* culture. However concentration of agar has affected to root

induction. High concentration of agar inhibited root development and handicapped root adsorbed water. Suthar *et al*. (2011) reported that high concentration of agar reduced root formation because agar blocked to uptake nutrients in medium.

Concentration of $AgNO_3$ on root and flower induction, Silver nitrate that break ethylene action inhibited root induction in plant though it promoted flower (Kumar *et al*., 2009). However, root induction of apple was encouraged by effect of $AgNO_3$ inhibited ethylene action (Ma *et al*., 1998). Chithra *et al*. (2004) recorded that AgNO3 promoted rooting and flowering in rare rhoeophytic woody medicinal plant whereas rooting of persian violet was interrupted by silver nitrate. $AgNO_3$ incited flower formation in some plant such as *Capsicum frutescens* (Sharma *et al*., 2008), *Rosa indica* (Pratheesh and Kumar, 2012).

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