Growth of Chrysanthemum Explants on MS Medium Sterilized by Disinfectants and Essential Oils

Wittaya Deein, Chockpisit Thepsithar, Aree Thongpukdee, and Suppaya Tippornwong

***Abstract*—A sterile condition of culture medium is one of the main aspects for micropropagation. The alternative technique for medium sterilization to replace autoclaving was carried out. For sterilization of Murashige and Skoog (MS) medium, commercial essential oils, disinfectants or in combinations were tested. Each essential oil or disinfectant or combination was added to a 20-mL medium in a 120-mL container, kept for 2 weeks before evaluating sterile conditions. Treated media were compared to control medium, autoclaved at 121 degree Celsius for 15 min. Sterile conditions of MS medium were found 100% from 10% povidone-iodine (108 μL), 6% sodium hypochlorite (36 μL), 2% iodine + 2.4% potassium iodide (36 μL), while 95% sterile conditions were obtained from 2% iodine + 2.4% potassium iodide in combination with 10% povidone-iodine (ratio 1 : 1 at 36 μL and ratio 1 : 3 at 72 μL), 10% povidone-iodine in combination with lemon oil (ratio 3 : 1, 108 μL) compared to 100% sterile conditions from autoclaved medium. Effects of these treated media on growth of chrysanthemum shoot and node explants were investigated. It was found that growth of explants on medium treated with 10% povidone-iodine or 2% iodine + 2.4% potassium iodide alone or in combination with 10% povidone-iodine (ratio 1 : 1) or 6% sodium hypochlorite was comparable to those on autoclaved medium.**

***Index Terms*—MS medium, chrysanthemum, disinfectants, essential oils, sterilization, plant tissue culture.**

I. Introduction

Plant tissue culture is an effective technique for plant micropropagation by using many parts of plants such as cells, tissues and organs, cultured on synthetic medium under sterile conditions. Unfortunately, most agriculturists cannot carry out plant tissue culture laboratory by themselves due to high production costs. One of the major problems is expensive equipment especially an autoclave, a sterilizing apparatus. Therefore, the development of techniques, using chemicals or plant extracts or in combinations for sterilizing culture medium, to replace the autoclaving method for establishing aseptic culture medium will be the optional procedure for plant tissue culture.

The use of disinfectants, fungicides, bactericides and biocides such as sodium hypochlorite, calcium hypochlorite, chlorine, methylchloroisothaiazolinone, hydrogen peroxide, and chemical mixtures containing methylisothiazolinone, magnesium chloride, magnesium nitrate, potassium sorbate and sodium benzoate supplemented in culture medium for preventing contamination was reported [1], [2]. Sterile culture media without autoclaving of some plants using sodium hypochlorite for sugarcane micropropagation [3] and sodium hypochlorite or sodium dichloroisocyanurate for orchid cultures [4]–[7] were established. Moreover, the studies on plant extracts and essential oils as microorganism inhibitors were also presented on clove (*Eugenia caryophyllata* Thunb.) [8], [9], lemon [*Citrus limon* (L.) Burm. F.] [10], [11], cinnamon (*Cinnamomum zeylanicum* Breyne) [12]–[14], betel (*Piper betle* L.) [15]–[17], cassumunar ginger (*Zingiber cassumunar* Roxb.) [18], holy basil (*Ocimum sanctum* L.), lavender (*Lavandula angustifolia* Mill.) [19], [20], bergamot (*C. bergamia* Risso) [21], and turmeric (*Curcuma longa* L.) [22].

This research reported effects of disinfectants and essential oils as sterilizing agents on sterile conditions of MS medium and growth of chrysanthemum shoot and node explants on treated medium.

II. Materials and Methods

A. Medium Used

The medium used for *in vitro* culture of chrysanthemum nodes was Murashige and Skoog (MS) medium [23] supplemented with 30 g/L sucrose and 5.5 g/L agar (Hardy Diagnostics Criterion agar, Bacteriological grade, USA). The pH of the medium was adjusted to 5.8. Each essential oil (cinnamon oil, clove oil, and lemon oil) or disinfectant [2% iodine + 2.4% potassium iodide (KI), 10% povidone-iodine and 6% sodium hypochlorite (NaOCl)] or combination was added in a 120-mL glass jar containing 20 mL of culture medium in various concentrations (9 – 180 μL). All media were kept in room temperature (29±2 ℃) for 2 weeks before investigating effects of sterilizing agents on sterile conditions of media compared to autoclaved medium. Numbers of containers containing culture medium that obtained totally sterile condition were collected. Sterilizing agents added in medium providing 95 – 100% sterile conditions were chosen for culturing shoot and node explants of chrysanthemum 'Moneymaker Improved', about 1 cm. long with 2 nodes, for 4 and 6 weeks, respectively.

B. Culture Conditions

All cultures were incubated under a 25±1 ℃ with a 16 h photoperiod at 35 – 40 μmole $m^{-2}s^{-1}$ provided by cool white lights.

Manuscript received May 7, 2013; revised July 15, 2013. This work was supported in part by Toray Science Foundation (TTSF) and Faculty of Science, Silpakorn University, Thailand.

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C. Statistical Analysis

Sterile conditions of media were evaluated after 2 weeks with 20 replications. Growth of chrysanthemum shoots from shoot and node explants, whole fresh weight (FW), shoot length, root length and number of node, were collected after 4 and 6 weeks of culturing, respectively. Each treatment was replicated 10 times. The completely randomized design (CRD) was used as the experimental design and means were compared by Duncan's New Multiple Range Test (DMRT) at $P = 0.05$ [24].

III. Results

A. Effects of Sterilizing Agents on Sterile Conditions of Treated Media

A 20-mL MS medium was treated with each sterilizing agent, kept in room temperature (about 29 ±2 ℃) for 2 weeks. For MS medium, 95 – 100% sterile conditions of culture medium was found from medium supplemented with clove oil at 9 µL/20 mL medium, cinnamon oil, 2% iodine + 2.4% KI, or 6% NaOCl at 36 µL/20 mL medium, and 10% povidone-iodine at 108 µL/20 mL medium. For sterilizing agents in combinations, 95 – 100% sterile conditions of culture medium was found from medium supplemented with 2% iodine + 2.4% KI : 10% povidone-iodine (1 : 1) at 36 µL/20 mL medium, 2% iodine + 2.4% KI : 10% povidone-iodine (1 : 3) or 2% iodine + 2.4% KI : clove oil at 72 µL/20 mL medium, 2% iodine + 2.4% KI : cinnamon oil (6 : 1) or 10% povidone-iodine : lemon oil (3 : 1) at 108 µL/20 mL medium (Table I).

TABLE I: Percentage of Sterile MS Medium after Treated with Different Essential Oils, Disinfectants, and Combinations for 2 Weeks

Treatment	Sterile condition (%) [1]					
	Concentration (µL) in a 20-mL MS					
	9	18	36	72	108	180
Autoclaved (control)	100					
Cinnamon oil (A)	35	40	100	100	100	100
Clove oil (B)	95	100	100	100	100	100
Lemon oil (C)	45	45	60	70	80	90
2% Iodine + 2.4% KI (D)	-	-	100	100	100	100
10% Povidone-iodine (E)	-	-	85	95	100	100
6% NaOCl (F)	-	-	100	100	100	100
D : E (1 : 1)	-	-	95	100	100	100
D : E (1 : 3)	-	-	55	95	100	100
D : A (6 : 1)	-	-	75	85	100	100
D : B (6 : 1)	-	-	50	95	100	100
D : C (3 : 1)	-	-	45	50	55	-
E : A (6 : 1)	-	-	45	55	80	-
E : B (6 : 1)	-	-	25	50	70	-
E : C (3 : 1)	-	-	30	75	95	100

[1] $n = 20$; - = not available

B. Effects of Sterilizing Agent-Treated Media on Growth of Chrysanthemum Explants

Medium supplemented with clove oil and cinnamon oils (9 and 36 µL/20 mL medium), disinfectants (36 and 108 µL/20 mL medium), and combinations (36, 72, and 108 µL/20 mL medium) that gave 95 – 100% sterile conditions of medium were chosen for culturing chrysanthemum shoot and node explants.

For growth of shoot explants, autoclaved medium provided 536.2 mg. FW, 3.12 cm. shoot length, 3.57 cm. root length, and 8.1 nodes (Fig. 1 a)). For disinfectants alone, 2% iodine + 2.4% potassium iodide and 6% NaOCl at 36 µL/20 mL medium obtained 311.8 and 229.3 mg. FW, 2.26 and 1.54 cm. shoot length, 4.11 and 5.31 cm. root length, 7.4 and 5.2 nodes, respectively (Fig. 1 b) and 1 d)). However, 10% povidone-iodine at 108 µL/20 mL medium gave the best results providing 555.6 mg. FW, 2.77 cm. shoot length, 5.73 cm. root length, and 7.4 nodes (Fig. 1 c)). For combinations of disinfectants, 2% iodine + 2.4% KI : 10% povidone-iodine (1 : 1) at 36 µL/20 mL medium and 2% iodine + 2.4% KI : 10% povidone-iodine (1 : 3) at 72 µL/20 mL medium provided 250.5 and 191.3 mg. FW, 1.92 and 1.39 cm. shoot length, 5.43 and 3.22 cm. root length, 8.3 and 7.4 nodes, respectively (Fig. 1 e) and Fig. 1 f)). A combination of disinfectant and essential oil, 10% povidone-iodine : lemon oil (3 : 1) at 108 µL/20 mL medium, gave 182.3 mg. FW, 1.24 cm. shoot length, 2.78 cm. root length, and 6.5 nodes (Fig. 1 g)). In other treated media, no growth was found and explants died eventually (Table II).

TABLE II: Growth of Chrysanthemum 'Moneymaker Improved' Shoots on Treated MS Medium after Culturing for 4 Weeks

Treatment in 20 mL medium		Growth of chrysanthemum shoot [2]			
Sterilizing agents [1]	µL	Fresh weight (g)	Shoot length (cm)	Root length (cm)	No. of node
Autoclaved	0	536.2 ±30.3 b	3.12 ±0.03 a	3.57 ±0.09 e	8.1 ±0.1 a
A	36	0.0 ±0.0 h	0.00 ±0.00 g	0.00 ±0.00 h	0.0 ±0.0 e
B	9	0.0 ±0.0 h	0.00 ±0.00 g	0.00 ±0.00 h	0.0 ±0.0 e
D	36	311.8 ±17.0 c	2.26 ±0.06 c	4.11 ±0.07 d	7.4 ±0.1 b
E	108	555.6 ±9.3 a	2.77 ±0.03 b	5.73 ±0.03 a	7.4 ±0.1 b
F	36	229.3 ±5.8 d	1.54 ±0.03 e	5.31 ±0.03 c	5.2 ±0.1 d
D : E (1 : 1)	36	250.5 ±10.3 e	1.92 ±0.06 d	5.43 ±0.03 b	8.3 ±0.1 a
D : E (1 : 3)	72	191.3 ±7.7 f	1.39 ±0.03 f	3.22 ±0.03 f	7.4 ±0.1 b
D : A (6 : 1)	108	0.0 ±0.0 h	0.00 ±0.00 g	0.00 ±0.00 h	0.0 ±0.0 e
D : B (6 : 1)	72	0.0 ±0.0 h	0.00 ±0.00 g	0.00 ±0.00 h	0.0 ±0.0 e
E : C (3 : 1)	108	182.3 ±11.1 g	1.24 ±0.03 f	2.78 ±0.07 g	6.5 ±0.1 c

[1] A = Cinnamon oil; B = Clove oil; C = Lemon oil; D = 2% Iodine + 2.4% KI; E = 10% Povidone-iodine; F = 6% NaOCl

[2] Values are means ($n = 10$). Means followed by the same letters within the same column are not significantly different at $P = 0.05$ by DMRT.

For growth of nodes explants, autoclaved medium provided 446.7 mg. FW, 3.47 cm. new shoot length, 6.43 cm. root length, and 7.3 nodes (Fig. 2 a)), while clove oil at 9 µL/20 mL medium gave 88.7 mg. FW, 0.67 cm. new shoot length, and 5.3 nodes (Fig. 2 b)), and 2% iodine + 2.4% KI and 6% NaOCl at 36 µL/20 mL medium obtained 314.7 and 387.7 mg. FW, 2.10 and 2.40 cm. new shoot length, 7.47 and 5.63 cm. root length, 7.7 and 8.0 nodes, respectively (Fig. 2 c) and Fig. 2 e)). However, 10% povidone-iodine at 108 µL/20 mL medium gave the best results obtaining 597.3 mg. FW, 3.40 cm. new shoot length, 7.87 cm. root length, and 9.0 nodes (Fig. 2 d)).

In addition, for growth of node explants on medium added with each of a combination of sterilizing agents, 2% iodine + 2.4% KI : 10% povidone-iodine (1 : 1) at 36 µL/20 mL medium and 2% iodine + 2.4% KI : 10% povidone-iodine (1 : 3) at 72 µL/20 mL medium gave 456.0 and 311.3 mg. FW, 2.73 and 1.93 cm. new shoot length, 7.83 and 7.37 cm. root length, 8.3 and 7.3 nodes, respectively (Fig. 2 f) and Fig. 2 g)). Whist, 10% povidone-iodine : lemon oil (3 : 1) at 108 µL/20 mL medium gave 246.7 g. FW, 1.40 cm. new shoot length, 7.33 cm. root length, and 6.7 nodes (Fig. 2 h)). In other treated media, the same results as shoot growth were obtained. No growth was found and explants died (Table III).

TABLE III: GROWTH OF CHRYSANTHEMUM 'MONEYMAKER IMPROVED' NODES ON TREATED MS MEDIUM AFTER CULTURING FOR 6 WEEKS

Treatment in 20 mL medium		Growth of chrysanthemum shoot [2]			
Sterilizing agents [1]	µL	Fresh weight (g)	Shoot length (cm)	Root length (cm)	No. of node
Autoclaved	0	446.7 ±17.1 b	3.47 ±0.12 a	6.43 ±0.15 c	7.3 ±0.3 bc
A	36	0.0 ±0.0 f	0.00 ±0.00 f	0.00 ±0.00 f	0.0 ±0.0 d
B	9	88.7 ±2.9 f	0.67 ±0.03 f	0.00 ±0.00 f	5.3 ±0.0 d
D	36	314.7 ±7.5 d	2.10 ±0.12 d	7.47 ±0.18 ab	7.7 ±0.3 bc
E	108	597.3 ±30.3 a	3.40 ±0.10 a	7.87 ±0.18 a	9.0 ±0.6 a
F	36	387.7 ±9.7 c	2.40 ±0.12 c	5.63 ±0.12 d	8.0 ±0.0 ab
D : E (1 : 1)	36	456.0 ±13.9 b	2.73 ±0.03 b	7.83 ±0.03 a	8.3 ±0.3 ab
D : E (1 : 3)	72	311.3 ±6.4 d	1.93 ±0.09 d	7.37 ±0.15 b	7.3 ±0.3 bc
D : A (6 : 1)	108	0.0 ±0.0 f	0.00 ±0.00 f	0.00 ±0.00 f	0.0 ±0.0 d
D : B (6 : 1)	72	0.0 ±0.0 f	0.00 ±0.00 f	0.00 ±0.00 f	0.0 ±0.0 d
E : C (3 : 1)	108	246.7 ±4.1 e	1.40 ±0.06 e	7.33 ±0.12 b	6.7 ±0.3 c

[1] A = Cinnamon oil; B = Clove oil; C = Lemon oil; D = 2% Iodine + 2.4% KI; E = 10% Povidone-iodine; F = 6% NaOCl

[2] Values are means (n = 10). Means followed by the same letters within the same column are not significantly different at $P=0.05$ by DMRT.

IV. DICUSSIONS

In the experiment, 10% povidone-iodine or 6% sodium hypochlorite or 2% iodine + 2.4% potassium iodide at the concentration of 108, 36, 36 µL/20 mL medium, respectively, were promising to use as sterilizing agents in solid MS medium. These disinfectants provided completely sterile condition of MS medium. The disinfectant-treated medium could be used for culturing chrysanthemum shoot and node explants. *In vitro* plantlets obtained from medium treated with these disinfectants showed similar or better growth than those obtained from autoclaved medium. The results were similar to the reports of Teixeira *et al.* [5], Yanagawa *et al.* [6] and Chansean and Syoichi [7]. Culture media, for wild orchid seeds germination, *Cymbidium* and *Phalaenopsis* micropropagation, were sterilized by adding sodium hypochlorite at the appropriate concentrations of 0.005% active chlorine [6], [7]. Teixeira *et al.* [5] reported that active chlorine at the concentrations of 0.0003% or 0.0005% provided completely sterile condition of culture medium for pineapple micropropagation. Moreover, sodium hypochlorite at the concentration of 0.1% was reported to be used as chemical sterilizing agent of culture medium for sugarcane without autoclaving [3], [25]. Cardoso and Teixeira da Silva [26] presented the effective chemical sterilization of medium using 0.0025% chlorine dioxide (ClO_2) for gerbera micropropagation.

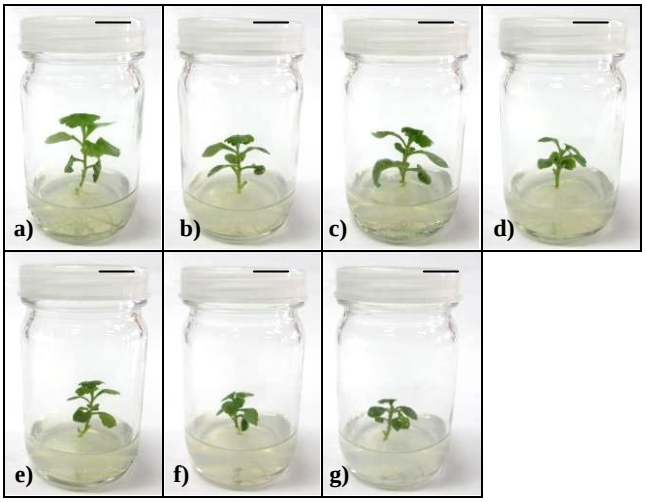

Fig. 1. Growth of chrysanthemum 'Moneymaker Improve' shoot explants on sterilizing agent treated 20-mL MS medium culture for 5 weeks (bar = 1 cm.) a) autoclaved medium; b) 36 µL 2% Iodine + 2.4% KI (D); c) 108 µL 10% povidone-iodine (E); d) 36 µL 6% NaOCl (F); e) 36 µL D : E = 1 : 1; f) 72 µL D : E = 1 : 3; g) 108 µL E : Lemon oil (C) = 1 : 3.

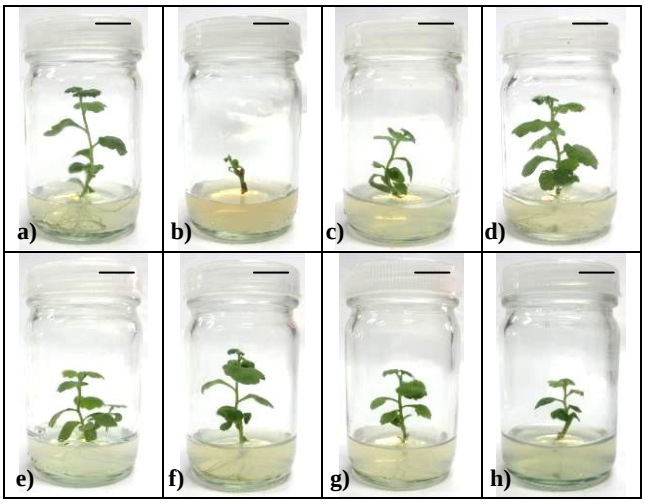

Fig. 2. Growth of chrysanthemum 'Moneymaker Improve' node explants on sterilizing agent treated 20-mL MS medium culture for 5 weeks (bar = 1 cm.) a) autoclaved medium; b) 9 µL clove oil (B); c) 36 µL 2% iodine + 2.4% KI (D); d) 108 µL 10% povidone-iodine (E); e) 36 µL 6% NaOCl (F); f) 36 µL D : E = 1 : 1; g) 72 µL D : E = 1 : 3; h) 108 µL E : Lemon oil (C) = 1 : 3.

Essential oils showed antimicrobial activity. Fungicidal efficacy against *Rhizoctonia solani*, *Aspergillus flavus* and *Fusarium verticillioides* was found from betel [16], [17]. Antibacterial activity against some food-borne pathogens, gram negative and/or gram positive bacteria was found from clove [8], betel [15], cassumunar ginger [18], lavender [19, 20], lemon and bergamot [21] and turmeric [22]. In the experiment, cinnamon oil and clove oil at the concentrations of 36 and 18 µL/20 mL medium provided completely sterile conditions of MS medium. However, these essential oils were toxic to chrysanthemum shoot and node explants and very poor growth or no survival of explants was found. For combinations of disinfectants and essential oils, it was found that 2% iodine + 2.4% KI : cinnamon oil (6 : 1) and 2% iodine + 2.4% KI : clove oil (6 : 1) at the concentrations of

108 and 72 µL/20 mL medium gave 100 and 95% sterile conditions of MS medium, respectively, but no growth of explants was found. However, growth of shoot and node explants was observed from medium treated with 10% povidone-iodine : lemon oil (3 : 1) at the concentration of 108 µL/20 mL medium with 95% sterile condition of medium.

The report on using disinfections, essential oils and in combinations as sterilizing agents was established to eliminate microorganisms in MS medium to obtain sterile condition without autoclaving. The treated medium could be used for culturing chrysanthemum apical shoots and nodes. However, further experiments are needed to establish appropriate concentrations of single essential oil or various combinations as sterilizing agents in MS medium used for plant tissue culture.

V. Conclusions

Disinfectants, 10% povidone-iodine or 2% iodine + 2.4% potassium iodide, at the appropriate concentrations (108 µL/20 mL medium and 36 µL/20 mL medium, respectively), were effective for eradicate microorganisms, causal agents of *in vitro* contamination, and provided completely sterile conditions of solid MS medium without autoclaving. MS medium treated with these sterilizing agents could be used for culturing chrysanthemum 'Moneymaker Improved' shoots and nodes. For essential oil alone added in MS medium, no growth of chrysanthemum shoot and node explants was found and explants died eventually. However, growth of explants was observed from medium added with 10% povidone-iodine in combination with lemon oil (ratio 3 : 1) at the concentration of 108 µL /20 mL medium) that provided 95% sterile condition of MS medium.

Acknowledgements

This research was financial supported by Thailand Toray Science Foundation (TTSF) and Faculty of Science, Silpakorn University, Thailand.

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