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Sterilisation of Hyponex Medium by Chemicals without Autoclaving and Growth of *Phalaenopsis* Protocorms

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***Abstract*—**For sterilization of *Phalaenopsis* culture medium without autoclaving, selected single sterilizing agents and in combinations were added to a 25ml Hyponex medium in a 120ml glass container. Treated liquid and solid media, supplemented with sterilizing agents, were compared to a control medium, autoclaved at 121°C for 15min. It was found that 90µL of 10% povidone-iodine, 150µL of 5.25% sodium hypochlorite, 150µL of 2% mercurochrome, 90µL of 2.5% iodine + 2.5% potassium iodine in combination with 10% providone-iodine (1:3) and 30µL of 2.5% iodine + 2.5% potassium iodide in combination with 2% mercurochrome showed 100% sterile conditions in liquid medium but provided 75, 100, 50, 75 and 80% sterile conditions, respectively, in solid medium. For growth of *Phalaenopsis* protocorms, 90µL of 10% povidone-iodine in liquid Hyponex medium gave the comparable growth of protocorms to control medium while 150µL of 5.25% sodium hypochlorite in solid medium provided the promising growth of protocorms. Growth of protocorms, whole fresh weight, numbers of leaf and root, root length and number of protocorm-like bodies, was discussed.

Keywords*—Phalaenopsis,* sterilizing agents, Hyponex medium, sterile medium without autoclaving.

I. Introduction

PLANT tissue culture is a very useful technology for plant propagation. The knowledge about micropropagatation by has been provided to agriculturists worldwide. Unfortunately, most agriculturists cannot carry out 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 tissue culture techniques, using chemicals or natural plant extracts or in combinations to eradicate all micro-organisms causing agents of contamination, to replace the autoclaving method for establishing aseptic culture medium will be the best procedure for plant tissue culture. The use of biocides, disinfectants, fungicides and bactericides such as sodium hypochlorite, calcium hypochlorite, hydrogen peroxide, chlorine, methylchloroisothaiazolinone 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 including orchids were reported using sodium hypochlorite or sodium dichloro- isocyanurate [3]-[6].

This study reports effective sterilizing agents supplemented in Hyponex medium to sterilize medium without autoclaving and growth of protocorms of *Phalaenopsis* 'Silky Moon' on sterilizing agent-treated media.

II. Materials and Methods

The medium used for *Phalaenopsis* protocorm growth was Hyponex medium [7] containing 3.5g/L Hyponex (6.5N-6P-19K), 20g/L sucrose as a liquid medium. For a solid medium, 1g/L activated charcoal and 2.2g/L Phytagel were added. The pH of the medium was adjusted to 5.4. Sterilizing agents, 10% povidone-iodine, 5.25% sodium hypochlorite, 2% merbromin solution or 0.1% acriflavin solution, were added in a 120ml glass jar containing 25ml of heated liquid or solid culture medium, in various concentrations (30 – 450µL) alone or in combinations with 2.5% iodine + 2.5% potassium iodide. All media were kept in room temperature (about 29±2°C) for 2 weeks to investigate effects of sterilizing agents on sterile conditions of media compared to autoclaved medium. Explants (protocorms of *Phalaenopsis* 'Silky Moon' about 2mg/protocorm) were cultured for 14 weeks on sterilizing agent-treated media, kept in room temperature 1 day before culturing. All cultures were incubated under a 24±1°C with a 16h photoperiod at 35-40µmole·$m^{-2}s^{-1}$ provided by cool white lights.

Sterile conditions of media were recorded after 2 weeks. For growth of protocorms, whole fresh weight, numbers of leaf and root, root length and number of protocorm-like bodies (PLBs) were collected after 14weeks of culturing. Each treatment was replicated 20 times. The completely randomized design (CRD) was used as the experimental design and means were compared by Duncan's New Multiple Range Test at P = 0.05 [8].

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III. Results

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

A 25ml culture medium was treated with each sterilizing agent, alone or in combinations, kept in room temperature (about 29±2°C) for 2 weeks. For liquid Hyponex medium, complete sterilization (100%) of culture medium was found from medium supplemented with 90µL of 10% povidone-iodine (A), 150µL of 5.25% sodium hypochlorite (B), 150µL of 2% merbromin solution (C), 90µL of a combination of 2.5% iodine + 2.5% potassium iodide (E) and A (1:3), 30µL of a combination of E and C (1:3) or 150µL of a combination of E and 0.1% acriflavin solution (D) (1:3). For solid Hyponex medium, less sterile conditions of media were found from the same concentrations of sterilizing agents, providing 75, 100, 50, 75, 80 and 90% sterile conditions, respectively. However, 100% sterile conditions of solid media were found from 210µL of A, 300µL of C, 150µL of E:A (1:3) and 90µL of E:C (1:3) (Table I).

B. Effects of Sterilizing Agent-Treated Media on Growth of Phalaenopsis "Silky Moon' Protocorms

The 25ml sterile treated media both solid and liquid medium were kept in room temperature for 1 day before culturing. Protocorms of *Phalaenopsis* 'Silky Moon' (about 2mg/protocorm) were cultured for 14 weeks on a treated medium, 5 protocorms per a container. For solid medium, no growth of protocorms was found on medium supplemented with 10% povidone-iodine (90µL), 2% merbromin solution (210µL) or a combination of E and A (30µL). However, 150µL of 5.25% sodium hypochlorite and 30µL of E:C (1:3) provided the best growth of protocorms with 18.8 – 19.5mg FWs, 2.2 leaves, 1.6 – 1.8 roots with 1.3 – 1.5cm long and 2.3 – 5.0 PLBs per explant. The control set (the autoclaved medium) provided 15.5mg FWs, 2.3 leaves, 1 root with 0.7cm long and 2.7 PLBs (Table II; Fig. 1). For liquid medium, the best growth of protocorms was found in medium supplemented with 90µL of 10% povidone-iodine, providing 11.5mg FWs, 1.4 leaves, 0.1 root with 0.05cm long and 1.6 PLBs compared to autoclaved medium with 13.4mg FWs, 0.7 leaf, 0.2 root with 0.06cm long and 1.4 PLBs (Table III; Fig. 2).

TABLE I
PERCENTAGE OF STERILE CONDITIONS OF HYPONEX MEDIUM AFTER TREATED WITH DIFFERENT STERILIZING AGENTS FOR 2 WEEKS

Sterilizing agents	Sterile conditions of treated media after 2 weeks (%) [1]											
	µL of sterilizing agents in 25-mL solid medium						µL of sterilizing agents in 25-mL liquid medium					
	30	90	150	210	300	450	30	90	150	210	300	450
Autoclaved	50	75	90	100	100	100	70	100	100	100	100	100
10% Povidone-iodine (A)	80	80	100	100	100	100	90	95	100	100	100	100
5.25% Sodium hypochlorite (B)	30	45	50	80	100	100	50	75	100	100	100	100
2% Merbromin solution[2] (C)	0	0	10	50	50	60	0	0	0	0	0	10
0.1% Acriflavin solution (D)	60	75	100	100	100	100	80	100	100	100	100	100
(2.5% iodine + 2.5% potassium iodide):A (1:3)	0	0	0	0	0	0	0	0	0	0	0	0
(2.5% iodine + 2.5% potassium iodide):B (1:3)	80	100	100	100	100	100	100	100	100	100	100	100
(2.5% iodine + 2.5% potassium iodide):C (1:3)	10	90	100	100	100	100	75	90	100	100	100	100
(2.5% iodine + 2.5% potassium iodide):D (1:3)	50	75	90	100	100	100	70	100	100	100	100	100

Fig. 1 Growth of *Phalaenopsis* 'Silky Moon' protocorm-like bodies on treated 25ml solid Hyponex medium cultured for 14 weeks
A = 150µL of 5.25% sodium hypochlorite; B = 30µL of 2.5% iodine + 2.5% potassium iodide: 2% merbromin solution (1:3); C = control (autoclaved medium)

TABLE II
GROWTH OF PROTOCORM-LIKE BODIES OF *PHALAENOPSIS* 'SILKY MOON' ON STERILIZING AGENT-TREATED SOLID HYPONEX MEDIUM AFTER CULTURING FOR 14 WEEKS

Treatments [1]	µL/25 mL medium	Growth of PLBs cultured on treated solid Hyponex medium/explants [3]				
		Whole FWs [2] (mg.)	No. leaves	No. root	Root length (cm.)	No. PLBs
Autoclaved	-	15.5±2.0 b	2.3±0.9 b	1.0±0.7 b	0.7 ±0.6 b	2.7±1.6 b
10% Povidone-iodine (A)	90	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
5.25% Sodium hypochlorite (B)	150	18.8±3.2 c	2.2±0.6 b	1.8±0.5 c	1.5±0.4 c	2.3±1.6 b
2% Merbromin solution (C)	210	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
(2.5% iodine + 2.5% potassium iodide):A (1:3)	30	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
(2.5% iodine + 2.5% potassium iodide):C (1:3)	30	19.5±2.8 c	2.2±0.4 b	1.6±0.6 c	1.3±0.5 c	5.0±2.5 c

[1] media were kept in the room temperature 1 day before culturing; [2] initial Fws of explants = 2.2 mg./protocorm;
[3] Values are mean ± SE (n = 20 with 5 explants/container). Means followed by the same letters within the same column are not significantly different at $P = 0.05$ by Duncan's new multiple range test.

TABLE III
GROWTH OF PROTOCORM-LIKE BODIES OF *PHALAENOPSIS* 'SILKY MOON' ON STERILIZING AGENT-TREATED LIQUID HYPONEX MEDIUM AFTER CULTURING FOR 14 WEEKS

Treatments [1]	µL/25 mL medium	Growth of PLBs cultured on treated liquid Hyponex medium/explants [3]				
		Whole FWs [2] (mg.)	No. leaves	No. root	Root length (cm.)	No. PLBs
Autoclaved	-	13.4±2.5 c	0.7±0.3 b	0.2±0.2 b	0.06±0.06 b	1.4±1.2 b
10% Povidone-iodine (A)	90	11.5±1.5 c	1.4±0.6 c	0.1±0.1 ab	0.05±0.05 ab	1.6±1.6 b
5.25% Sodium hypochlorite (B)	150	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.00±0.00 a	0.0±0.0 a
2% Merbromin solution (C)	210	7.4±1.8 b	0.0±0.0 a	0.0±0.0 a	0.00±0.00 a	0.0±0.0 a
(2.5% iodine + 2.5% potassium iodide):A (1:3)	30	7.6±2.0 b	0.5±0.3 b	0.0±0.0 a	0.03±0.03 ab	1.7±1.6 b
(2.5% iodine + 2.5% potassium iodide):C (1:3)	30	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.00±0.00 a	0.0±0.0 a

[1] media were kept in the room temperature 1 day before culturing; [2] initial Fws of explants = 2.2 mg./protocorm;
[3] Values are mean ± SE (n = 20 with 5 explants/container). Means followed by the same letters within the same column are not significantly different at $P = 0.05$ by Duncan's new multiple range test.

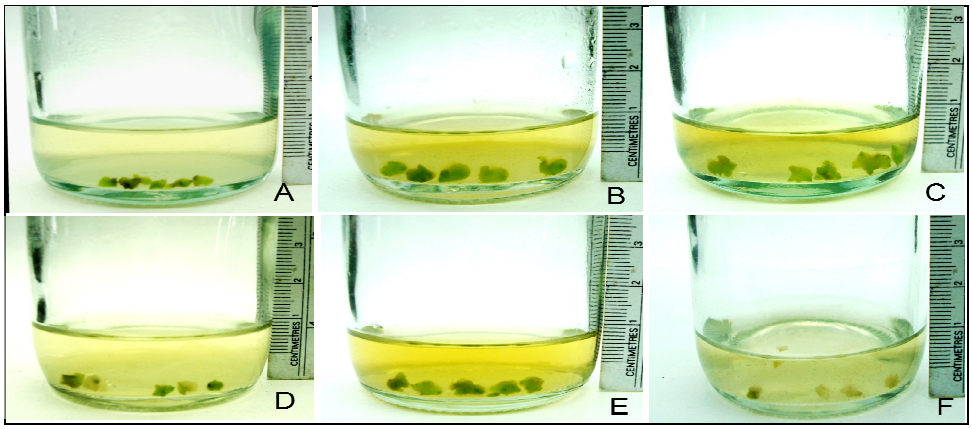

Fig. 2 Growth of *Phalaenopsis*'Silky Moon' protocorms on treated 25-mL liquid Hyponex medium cultured for 14 weeks
A = initial explants (a protocorm-like body about 2 mg) with 5 protocorms per container; B = control (autoclaved medium); C = 90 µL of 10% povidone-iodine, D = 210 µL of 2% merbromin solution; E = 30 µL of 2.5% iodine + 2.5% potassium iodide: 10% povidine-iodine (1:3); F = dead protocorms

IV. DISCUSSIONS

In this experiment, 5.25% sodium hypochlorite was chosen to use as sterilizing agent in solid Hyponex medium while 10% povidone-iodine was chosen to supplement in liquid Hyponex medium for culturing protocorms of *Phalaenopsis* 'Silky Moon'. The results were similar to the reports of Teixeira et al. [4], Yanagawa et al. [5] and Chansean and Syoichi [6]. Culture media, for some wild orchid seeds germination, *Cymbidium* and *Phalaenopsis* micropropagation, were sterilized by adding sodium hypochlorite solution at the appropriate concentrations of 0.005% active chlorine [5], [6]. Teixeira et al. [4] reported that active chlorine at the concentrations of 0.0003% or 0.0005% provided complete sterilization of culture medium for pineapple micropropagation.

V. CONCLUSIONS

Sodium hypochlorite, povidone-iodine and a mixture of iodine + potassium iodide and merbromin solution were effective chemicals for eradicate microorganisms, causal agents of *in vitro* contamination, and provided completely sterile condition of Hyponex medium. The use of sodium hypochlorite or mixture of iodine + potassium iodide and merbromin solution added in solid Hyponex medium and povidone-iodine added in liquid Hyponex medium provided

the comparable growth of *Phalaenopsis* hybrid to growth on autoclaved medium.

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