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Sterilisation of *in vitro* Culture Medium of Chrysanthemum by Plant Essential Oils without Autoclaving

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Abstract**—**The alternative technique for sterilization of culture medium to replace autoclaving was carried out. For sterilization of culture medium without autoclaving, some commercial pure essential oils, bergamot oil, betel oil, cinnamon oil, lavender oil and turmeric oil, were tested alone or in combinations with some disinfectants, 10% povidone-iodine and 2% iodine + 2.4% potassium iodide. Each essential oil or combination was added to 25-mL Murashige and Skoog (MS) medium before medium was solidified in a 120-mL container, kept for 2 weeks before evaluating sterile conditions. Treated media, supplemented with essential oils, were compared to control medium, autoclaved at 121 degree Celsius for 15 min. *In vitro* sterile conditions were found 20 – 100% from these treated media compared to 100% sterile condition from autoclaved medium. Treated media obtained 100% sterile conditions were chosen for culturing chrysanthemum shoots. It was found that 10% povidone-iodine in combination with cinnamon oil (3:1) and 2% iodine + 2.4% potassium iodide in combination with lavender oil (1:3) at the concentration of 36 µL/25 mL medium provided the promising growth of shoot explants.

Keywords**—**Sterilizing agents, essential oils, disinfectants, MS medium, *in vitro* culture, chrysanthemum, sterilization of medium without autoclaving

I. Introduction

PLANT tissue culture is a useful technology for plant propagation. The knowledge has been provided to agriculturists worldwide. 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 essential oils or in combinations for eradicating microorganisms causing agents of contamination, to replace the autoclaving method for establishing sterile culture medium will be the best procedure for *in vitro* culture.

The use of disinfectants, fungicides and bactericides such as chlorine, sodium hypochlorite, calcium hypochlorite, hydrogen peroxide, methylchloroisothaiazo-linone, and chemical mixtures containing methylisothiazo-linone, 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 were reported including orchids [3], [4], [5], [6], [7], [8] using sodium hypochlorite, sodium dichloroisocyanurate or povidone-iodine, sugarcane using sodium hypochlorite [9], and chrysanthemum using 2% iodine + 2.4% potassium iodide, 10% povidone-iodine or 6% sodium hypochlorite [10], [11] . The studies on plant extracts and essential oils as micro-organism inhibitors were reported on betel (*Piper betle* L.) [11], [12], [13], [14], cassumunar ginger (*Zingiber cassumunar* Roxb.) [15], holy basil (*Ocimum sanctum* L.) and clove (*Eugenia caryophyllata* Thunb.) [16], lavender (*Lavandula angustifolia* Mill.) [17], [18], lemon [*Citrus limon* (L.) Burm. F.] and bergamot (*C. bergamia* Risso) [11], [19] and turmeric (*Curcuma longa* L.) [20].

This research reported effects of plant essential oils alone or in a combination with disinfectants on sterile condition of MS medium and growth of chrysanthemum shoots on treated medium.

II. Materials And Methods

A. Medium Used

The medium used for *in vitro* culture of chrysanthemum shoots was Murashige and Skoog (MS) medium [21] 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 (bergamot oil, betel oil, cinnamon oil, lavender oil and turmeric oil) alone and in combination with a disinfectant (10% povidone-iodine or 2% iodine + 2.4% potassium iodide) were added in a 120-mL glass jar containing 25 mL of heated culture medium in various concentrations (18 – 324 µL). 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. Number of medium containers that obtained total sterile medium was collected. Then sterilizing agents that provided 100% sterile conditions of medium were chosen for culturing shoot explants. Shoots of chrysanthemum 'Moneymaker Improved', about 0.5 – 0.7 cm. long with 1 node, were cultured on sterilizing agent-treated media for 5 weeks.

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B. Culture Conditions

All cultures were incubated under a 24±1°C with a 16 h photoperiod at 35 – 40 µmole·$m^{-2}s^{-1}$ provided by cool white lights.

C. Statistical Analysis

Sterile conditions of media were evaluated after 2 weeks with 20 replications. Growth of shoots from chrysanthemum shoot explants, whole fresh weight (FWs), shoot length, number of leaves and roots were collected after 5 weeks of culturing. 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 at $P = 0.05$ [22].

III. Results

A.Effects of Essential Oils on Sterile Conditions of Treated Media

A 25-mL MS medium was treated with each essential oil, kept in room temperature (about 29 ± 2°C) for 2 weeks. For MS medium, complete sterilization (100%) of culture medium was found from medium supplemented with betel oil and cinnamon oil at the concentration of 36 µL/25 mL medium as well as bergamot oil and lavender oil at the concentration of 108 µL/25 mL medium. For turmeric oil, high concentration at 324 µL/25 mL medium gave 100% sterile conditions of medium (Table I).

B.Effects of Essential Oil in Combination with Disinfectants on Sterile Conditions of Treated Media

For combinations of essential oils (cinnamon oil, betel oil and lavender oil chosen from Table I) and disinfectants (10% povidone-iodine or 2% iodine+2.4% KI), MS medium added with each combination at the concentrations of 36 and 108 µL/25 mL medium. It was found that 10% povidone-iodine:cinnamon oil (3:1), 2% iodine+2.4% KI:cinnamon oil (1:3) and 2% iodine+2.4% KI:lavender oil (1:3) at the concentration of 36 µL/25 mL medium showed 100% sterile conditions of culture medium (Table II).

C.Effects of Sterilizing Agent-Treated Media on Growth of Chrysanthemum Shoots

For each essential oil, growth of a chrysanthemum shoot explant was observed only from medium containing cinnamon oil (36 µL/25 mL medium), providing 0.22 g. FWs, 1.89 cm. shoot length with 2.3 leaves and 0.8 roots. For disinfectant in combination with essential oil, it was found that 10% povidone-iodine:cinnamon oil (3:1) and 2% iodine+2.4% KI:lavender oil (1:3) at the concentration of 36 µL/25 mL medium (Table III; Fig. 1 (b), 1 (e)) provided high growth of a shoot explant (0.54 – 0.61 g. Fws, 2.48 – 2.58 cm. shot length, 10.8 – 11.0 leaves and 11.0 – 18.8 roots) comparing to control medium (0.96 g. FWs, 3.03 cm. shoot length, 10.7 leaves and 14.7 roots) (Table III; Fig. 1 (a)). (Table III). Growth of shoot explants was not different (0.21 – 0.37 g. FWs, 1.90 – 2.08 cm. shoot length, 8.5 – 10.8 leaves and 7.8 – 11.0 roots) (Table III, Fig. 1 (c), 1 (d)) obtained from medium containing 10% povidone-iodine:cinnamon oil (3:1) at 72 µL/25 mL medium, 2% iodine+2.4% KI:cinnamon oil (1:3) at 36 µL/25 mL medium and 2% iodine+2.4% KI:lavender oil (1:3) at 72 µL/25 mL medium. No growth of shoot explants was observed on others essential oil and combinations (Table III).

TABLE I
PERCENTAGE OF STERILE MS MEDIUM AFTER TREATED WITH DIFFERENT ESSENTIAL OILS FOR 2 WEEKS

Treatments	Sterile conditions (%) [1]					
	Concentration (µL) in a 25-mL MS					
	18	36	108	180	252	324
Autoclaved	100					
Bergamot oil	NA	20	100	100	100	100
Betel oil	90	100	100	100	100	100
Cinnamon oil	NA	100	100	100	100	100
Lavender oil	50	70	100	100	100	100
Turmeric oil	NA	NA	40	70	90	100

[1] n = 20; NA = not available

TABLE II
PERCENTAGE OF STERILE MS MEDIUM AFTER TREATED WITH DIFFERENT COMBINATIONS OF DISINFECTANTS AND ESSENTIAL OILS FOR 2 WEEKS

Treatments	Sterile conditions(%) [1]	
	(µL) ina 25-mL MS	
	36	108
Autoclaved	100	
10% povidone-iodine: Cinnamon oil (3:1)	100	100
10% povidone-iodine: Lavender oil (3:1)	30	60
10% povidone-iodine: Betel oil (10:1)	30	80
2% iodine+2.4% KI: Cinnamon oil (1:3)	100	100
2% iodine+2.4% KI: Lavender oil (1:3)	100	100

[1] n = 20

IV. Discussions

In the experiment, 10% povidone-iodine:cinnamon oil (3:1) and 2% iodine+2.4% KI:lavender oil (1:3) at the concentration of 36 µL/25 mL medium were promising to use as sterilizing agents in solid MS medium for chrysanthemum shoot explants. These disinfectants provided completely sterile condition of MS medium. The results were similar to the reports of Teixeira et al. [4], Yanagawa et al. [5] and Chansean and Syoichi [6]. Culture media for wild orchid seed 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 micro-propagation. 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 [8]. For culturing *Phalaenopsis* and chrysanthemum, Clorox® (150 µL/25 mL medium) and Betadine® (30 – 90 µL/25 mL

medium) were used as sterilizing agents in VW medium and MS medium, respectively [10]. Deein et al. [11] presented that 2% iodine + 2.4% potassium iodide, 10% povidone-iodine or 6% sodium hypochlorite, at 36 µL/20 mL medium, were effective for eradicate causal agents of *in vitro* contamination, and provided completely sterile condition of solid MS medium without autoclaving. MS medium treated with these sterilizing agents can be used for culturing chrysanthemum 'Moneymaker Improved' nodes. For essential oils, growth of chrysanthemum nodes was found only on MS medium added with betel oil (9 µL/20 mL medium) and lemon oil (180 µL/20 mL medium) that provided 90% sterile condition of MS medium [11].

TABLE III
GROWTH OF SHOOTS FROM CHRYSANTHEMUM 'MONEYMAKER IMPROVED' SHOOT EXPLANTS ON TREATED MS MEDIUM AFTER CULTURING FOR 5 WEEKS

Treatment (µL/25 mL medium)		Growth of chrysanthemum shoot explant [2]			
Sterilising agent	amount (µL)[1]	Whole FWs (g)	Shoot length (cm)	No. leaves	No. of roots
Autoclaved	-	0.96±0.04 a	3.03±0.10 a	10.7±0.7 a	14.7±1.1 b
Bergamot oil	108	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
Bergamot oil	180	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
Betel oil	36	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
Betel oil	108	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
Cinnamon oil	36	0.22±0.09 cd	1.89±0.51 n	2.3±0.4 c	0.8±0.4 h
Cinnamon oil	72	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
Lavender oil	108	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
Lavender oil	180	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
10% povidone-iodine: Cinnamon oil (3:1)	36	0.61±0.04 b	2.48±0.17 b	11.0±0.4 a	18.5±0.8 a
10% povidone-iodine: Cinnamon oil (3:1)	72	0.25±0.03 cd	1.90±0.06 c	8.5±0.8 b	8.3±1.2 d
2% iodine+2.4% KI:Cinnamon oil (1:3)	36	0.21±0.02 d	2.05±0.03 bc	8.8±0.9 b	7.8±1.2 d
2% iodine+2.4% KI:Cinnamon oil (1:3)	72	0.00±0.00 e	0.00±0.00 d	0.0±0.0 d	0.0±0.0 e
2% iodine+2.4% KI:Lavender oil (1:3)	36	0.54±0.15 b	2.58±0.39 ab	10.8±0.4 a	11.0±1.2 c
2% iodine+2.4% KI:Lavender oil (1:3)	72	0.37±0.09 c	2.08±0.13 cb	10.1±1.0 ab	15.3±2.4 b

[1] amount of essential oils or disinfectants (µL) added in a 25-mL MS medium per container
[2] Values are mean ± SE (n = 10). 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

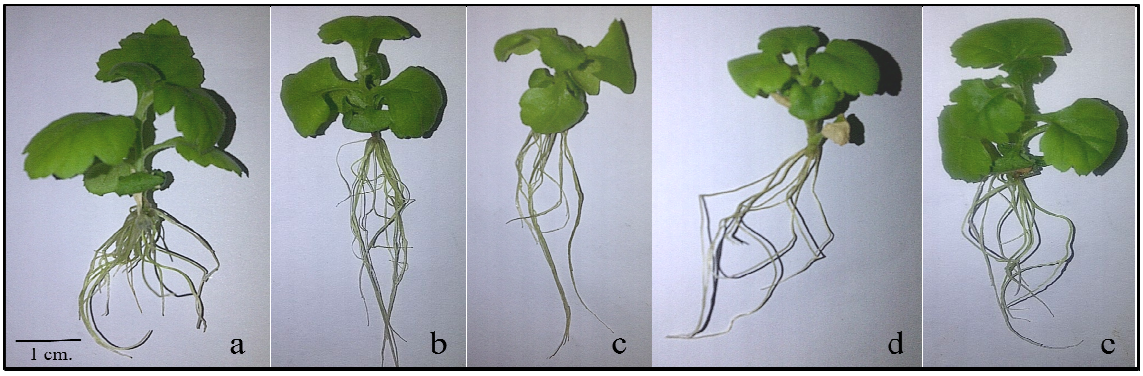

Fig. 1 Growth of a new shoot from chrysanthemum shoot explant on sterilizing agent treated 20-mL MS medium cultured for 5 weeks (bar = 1 cm.) a = autoclaved medium (control); b = 36 µL of 10% povidone-iodine:Cinnamon oil (3:1); c = 72 µL of 10% povidone-iodine:Cinnamon oil (3:1); d = 36 µL of 2% iodine+2.4% KI:Cinnamon oil (1:3); e = 36 µL of 2% iodine+2.4% KI:Lavender oil (1:3)

Plant extracts and plant essential oils showed antimicrobial activity. Fungicidal efficacy against *Rhizoctonia solani*, *Aspergillus flavus* and *Fusarium verticillioides* was found from betel [13], [14]. Antibacterial activity against some food-

borne pathogens, gram negative and/or gram positive bacteria was found from betel [12], lavender [17], [18] bergamot [19] and cinnamon [23]. For essential oils, growth shoots from chrysanthemum shoot explants was observed from MS medium added with cinnamon oil at 36 µL/25 mL medium. No growth of chrysanthemum shoot explants was found from medium containing other single essential oils. However, growth of shoot explants was observed from disinfectants in combinations with essential oils, 10% povidone-iodine: cinnamon oil (3:1), 2% iodine+2.4% KI:cinnamon oil (1:3) and 2% iodine+2.4% KI:lavender oil (1:3). Similar results were reported from Deein et al. [11].

The report showed the efficiency of plant essential oils in combinations of disinfectants as sterilising agents to prevent and eliminate microorganisms in MS medium to obtain sterile condition without autoclaving. 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

Among sterilising agents that provided 100% sterile conditions of MS medium, 10% povidone-iodine: Cinnamon oil (3:1) and 2% iodine+2.4% KI:lavender oil (1:3) at the appropriate concentration (36 µL/25 mL medium) were effective for eradicate microorganisms, causal agents of *in vitro* contamination, and provided completely sterile condition of solid MS medium without autoclaving. Moreover these sterilising agents added in Ms medium can be used for culturing chrysanthemum 'Moneymaker Improved' shoots and the growth of shoots were comparable to those obtained from autoclave medium.

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