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# *In vitro* Culture Medium Sterilization by Chemicals and Essential Oils without Autoclaving and Growth of Chrysanthemum Nodes

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***Abstract*****—**Plant tissue culture is an important *in vitro* technology applied for agricultural and industrial production. A sterile condition of culture medium is one of the main aspects. The alternative technique for medium sterilization to replace autoclaving was carried out. For sterilization of plant tissue culture medium without autoclaving, ten commercial pure essential oils and 5 disinfectants were tested. Each essential oil or disinfectant was added to a 20-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 or disinfectants, were compared to control medium, autoclaved at 121 degree Celsius for 15 min. Sterile conditions of MS medium were found 100% from betel oil or clove oil (18 μL/20 mL medium), cinnamon oil (36 μL/20 mL medium), lavender oil or holy basil oil (108 μL/20 mL medium), and lemon oil or tea tree oil or turmeric oil (252 μL/20 mL medium), compared to 100% sterile condition from autoclaved medium. For disinfectants, 2% iodine + 2.4% potassium iodide, 2% merbromine solution, 10% povidone-iodine, 6% sodium hypochlorite or 0.1% thimerosal at 36 μL/20 mL medium provided 100% sterile conditions. Furthermore, growth of new shoots from chrysanthemum node explants on treated media (fresh weight, shoot length, root length and number of node) were also reported and discussed in the comparison of those on autoclaved medium.

***Keywords*****—**Chrysanthemum, disinfectants, essential oils, MS medium, sterilizing agents, sterilization of medium without autoclaving.

## I. Introduction

PLANT tissue culture is a very 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 extracts or in combinations for eradicating microorganisms 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 chlorine, sodium hypochlorite, calcium hypochlorite, hydrogen peroxide, 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 dichloroisocyanurate [3]-[6]. The studies on plant extracts and essential oils as microorganism inhibitors were reported on betel (*Piper betle* L.) [7]-[9], cassumunar ginger (*Zingiber cassumunar* Roxb.) [10], holy basil (*Ocimum sanctum* L.) and clove (*Eugenia caryophyllata* Thunb.) [11], lavender (*Lavandula angustifolia* Mill.) [12], [13], lemon [*Citrus limon* (L.) Burm. F.] and bergamot (*C. bergamia* Risso) [14] and turmeric (*Curcuma longa* L.) [15].

This research reported effects of disinfectants and plant essential oils as sterilizing agents on sterile condition of MS medium and growth of chrysanthemum nodes 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 [16] 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, cassumunar ginger oil, cinnamon oil, clove oil, holy basil oil, lavender oil, lemon oil, tea tree oil and turmeric oil) or disinfectant (0.1% thimerosal, 2% merbromine solution, 2% iodine + 2.4% potassium iodide, 10% povidone-iodine and 6% sodium hypochlorite) was added in a 120-mL glass jar containing 20 mL of heated culture medium in various concentrations (9 – 396 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. Node explants of chrysanthemum 'Moneymaker Improved', about 1 cm. long with 2 nodes 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 a new shoot from a chrysanthemum node, whole fresh weight (FW), shoot length, root length and number of root 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 $P = 0.05$ [17].

## 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°C) for 2 weeks. For MS medium, complete sterilization (100%) of culture medium was found from medium supplemented with bergamot oil, cassumunar ginger oil, lemon oil, tea tree oil or turmeric oil at 252 µL/20 mL medium, betel oil or clove oil at 18 µL/20 mL medium, cinnamon oil, 2% iodine + 2.4% potassium iodide, 2% merbromin solution, 10% povidone-iodine, 6% sodium hypochlorite or 0.1% thimerosal at 36 µL/20 mL medium and holy basil or lavender oil at 108 µL/20 mL medium. However, 85 – 95% sterile conditions of medium were found from bergamot oil, holy basil oil or lavender oil at 36 µL/20 mL medium, betel oil or clove oil at 9 µL/20 mL medium, cassumunar ginger oil at 18 µL/20 mL medium, lemon oil at 180 µL/20 mL medium and tea tree oil at 108 µL/20 mL medium (Table I).

### *B. Effects of Sterilizing Agent-Treated Media on Growth of Chrysanthemum Nodes*

Medium supplemented with disinfectants (36 and 108 µL/20 mL medium) and essential oils (the concentrations that gave 85 – 100% sterile conditions of medium) were chosen for culturing chrysanthemum nodes. It was found that there was no growth and explants died on media added with bergamot oil, cassumunar ginger oil, holy basil oil, tea tree oil, clove oil (18 µL), lavender oil (108 µL), 2% merbromin solution and 0.1% thimerosal (Table II).

For essential oils, betel oil at 9 µL/20 mL medium and lemon oil at 180 µL/20 mL medium (providing 90% sterile conditions of medium) gave 0.252 and 0.180 g. FW, 2.11 and 1.89 cm. shoot length, 5.12 and 8.19 cm. root length with 5.5 and 5.3 nodes, respectively (Table II; Figs. 1 (g) and (i)). Other essential oils (providing 100% sterile conditions) gave 0.060 – 0.105 g. FW, 0.10 – 0.92 cm. shoot length, 0.00 – 2.66 cm. root length and 1.0 – 4.5 nodes (Table II; Figs. 1 (h) and (j)). Some essential oils, lavender oil and clove oil (providing 85 and 95% sterile conditions) gave 0.155 and 0.115 g. FW, 1.72 and 0.70 cm. shoot length, 6.89 and 2.29 cm. root length with 7.1 and 3.5 nodes, respectively (Table II; Figs. 1 (k) and (l)).

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

| Treatments | Sterile conditions (%) [1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Concentration (µL) in a 20-mL MS medium | | | | | | | |
| | 9 | 18 | 36 | 108 | 180 | 252 | 324 | 396 |
| Autoclaved | 100 | | | | | | | |
| Bergamot oil | 35 | 65 | 85 | 85 | 85 | 100 | - | - |
| Betel oil | 90 | 100 | 100 | 100 | 100 | 100 | - | - |
| Cassumunar ginger oil | 75 | 85 | 90 | 90 | 95 | 100 | - | - |
| Cinnamon oil | 35 | 40 | 100 | 100 | 100 | 100 | - | - |
| Clove oil | 95 | 100 | 100 | 100 | 100 | 100 | - | - |
| Holy basil oil | 20 | 65 | 95 | 100 | 100 | 100 | - | - |
| Lavender oil | 20 | 40 | 85 | 100 | 100 | 100 | - | - |
| Lemon oil | 45 | 45 | 60 | 80 | 90 | 100 | - | - |
| Tea tree oil | 35 | 35 | 45 | 95 | 95 | 100 | - | - |
| Turmeric oil | 10 | 20 | 45 | 60 | 75 | 100 | - | - |
| 0.1% Thimerosal | - | - | 100 | 100 | 100 | 100 | 100 | 100 |
| 2% Merbromin solution | - | - | 100 | 100 | 100 | 100 | 100 | 100 |
| 2% Iodine+2.4% KI | - | - | 100 | 100 | 100 | 100 | 100 | 100 |
| 6% NaOCl | - | - | 100 | 100 | 100 | 100 | 100 | 100 |
| 10% povidone-iodine | | | 100 | 100 | 100 | 100 | 100 | 100 |

N = 20

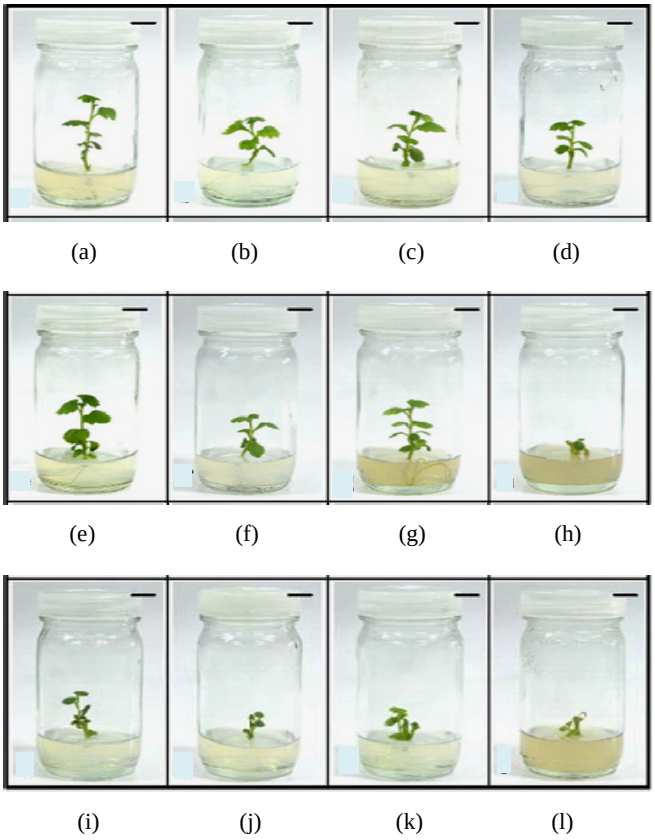


Fig. 1 Growth of a new shoot from chrysanthemum node explant on sterilizing agent treated 20-mL MS medium cultured for 5 weeks (bar = 1 cm.) (a) = autoclaved medium (control); (b) = 36 µL of 6% sodium hypochlorite; (c) = 36 µL of 2% iodine + 2.4% potassium iodide; (d) = 108 µL of 2% iodine + 2.4% potassium iodide; (e) = 36 µL of 10% povidone-iodine; (f) = 108 µL of 10% povidone-iodine; (g) = 9 µL of betel oil; (h) = 18 µL of betel oil; (i) = 180 µL of lemon oil; (j) = 252 µL of lemon oil; (k) = 36 µL of lavender oil; (l) = 9 µL of clove oil

TABLE II
GROWTH OF A NEW SHOOT FROM A CHRYSANTHEMUM 'MONEYMAKER IMPROVED' NODE EXPLANT ON TREATED MS MEDIUM AFTER CULTURING FOR 5 WEEKS

| Treatment (µL/20 mL medium) | | Growth of chrysanthemum node [2] | | | |
|---|---|---|---|---|---|
| Sterilizing agent | amount (µL)[1] | Fresh weight (g) | Shoot length (cm) | Root length (cm) | No. of node |
| Autoclaved | - | 0.342±0.003 a | 2.88±0.03 a | 7.41±0.02 f | 5.5±0.2 c |
| Bergamot oil | 252 | 0.000±0.000 m | 0.00±0.00 n | 0.00±0.00 n | 0.0±0.0 h |
| Betel oil | 9 | 0.252±0.002 d | 2.11±0.03 d | 5.12±0.02 i | 5.5±0.2 c |
| Betel oil | 18 | 0.105±0.002 i | 0.92±0.02 i | 2.66±0.05 j | 2.5±0.2 f |
| Cassumunar ginger oil | 95 | 0.000±0.000 m | 0.00±0.00 n | 0.00±0.00 n | 0.0±0.0 h |
| Cinnamon oil | 36 | 0.045±0.002 l | 0.10±0.00 m | 0.00±0.00 n | 1.0±0.0 g |
| Clove oil | 9 | 0.115±0.002 k | 0.70±0.03 k | 2.29±0.03 l | 3.5±0.2 e |
| Lavender oil | 36 | 0.155±0.002 g | 1.72±0.03 f | 6.89±0.02 g | 7.1±0.2 a |
| Lemon oil | 180 | 0.180±0.001 ef | 1.89±0.02 e | 8.19±0.03 e | 5.3±0.2 c |
| Lemon oil | 252 | 0.075±0.002 j | 0.82±0.03 j | 2.47±0.02 k | 4.5±0.2 d |
| Holy basil oil | 108 | 0.000±0.000 m | 0.00±0.00 n | 0.00±0.00 n | 0.0±0.0 h |
| Tea tree oil | 108 | 0.000±0.000 m | 0.00±0.00 n | 0.00±0.00 n | 0.0±0.0 h |
| Turmeric oil | 252 | 0.060±0.000 k | 0.21±0.02 l | 0.39±0.03 m | 3.6±0.2 e |
| 2% iodine+2.4% KI | 36 | 0.306±0.002 b | 2.22±0.03 c | 11.03±0.04 c | 7.2±0.2 a |
| 2% iodine+2.4% KI | 108 | 0.176±0.002 f | 1.62±0.03 g | 13.51±0.02 b | 6.1±0.2 b |
| 2% merbromine solution | 36 | 0.000±0.000 m | 0.00±0.00 n | 0.00±0.00 n | 0.0±0.0 h |
| 10% povidone-iodine | 36 | 0.345±0.002 a | 2.52±0.03 b | 9.12±0.03 d | 6.9±0.2 a |
| 10% povidone-iodine | 108 | 0.185±0.002 e | 1.52±0.03 h | 17.12±0.04 a | 5.5±0.2 c |
| 6% NaOCl | 36 | 0.290±0.002 c | 1.90±0.03 e | 6.13±0.03 h | 5.9±0.3 cd |
| 0.1% thimerosal | 36 | 0.000±0.000 m | 0.00±0.00 n | 0.00±0.00 n | 0.0±0.0 h |

[1] amount of essential oils or disinfectants ((µL) added in 20-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.

## IV. DISCUSSIONS

In the experiment, 10% povidone-iodine or 2% iodine + 2.4% potassium iodide or 6% sodium hypochlorite at 36 µL/20 mL medium 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 node explants. 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.

Plant extracts and plant essential oils showed antimicrobial activity. Fungicidal efficacy against *Rhizoctonia solani*, *Aspergillus flavus* and *Fusarium verticillioides* was found from betel [8], [9]. Antibacterial activity against some food-borne pathogens, gram negative and/or gram positive bacteria was found from betel [7], cassumunar ginger [10], clove [11], lavender [12], [13], lemon and bergamot [14] and turmeric [15]. For essential oils, growth of a new shoot from a chrysanthemum node explant was observed from MS medium added with betel oil at 9 µL/20 mL medium or lemon oil at 180 µL/20 mL medium. However, the concentrations of these two essential oils provided only 90% sterile condition of MS medium. Very poor growth or no growth of chrysanthemum nodes was found from essential oils at the concentrations that provided completely sterile condition of MS medium.

This was the first report on using plant essential oils as sterilizing agents to 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. CONCLUSION

Disinfectants, 2% iodine + 2.4% potassium iodide, 10% povidone-iodine or 6% sodium hypochlorite at the appropriate concentrations (36 µL/20 mL medium), were effective for eradicate microorganisms, 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.

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