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Formulasi Sediaan Sabun Cuci Tangan Ekstrak Jeruk Nipis (Citrus aurantifolia) dan Efektivitasnya sebagai Antiseptik

103

Uji Aktivitas Antioksidan Ekstrak Etanol Kayu Secang (Caesalpinia sappan) Menggunakan Metode DPPH, ABTS, dan FRAP

Cost Saving of Stress Ulcer Prophylaxis Used in Non-Intensive Care Unit (ICU) Inpatients

Hening Pratiwi, Laksmi Maharani, Ika Mustikaningtias 37-43

Abstract views: 8 PDF Downloads: 16 DOI: https://doi.org/10.24123/mpi.v3i1.2323

PDF

Profil Penggunaan Antibiotik Pada Pasien Pediatri Rujukan Berdasarkan Sistem Defined Daily Dose (DDD)

Nurul Fatmawati Pua Upa, Widyati Widiaty, Frans O.H. Prasetyadi 44-51

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PDF

Efektivitas Profilaksis Primer Filgrastim Pada Pasien Kanker Payudara terhadap Insiden Neutropenia

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Sirih Merah (Piper crocatum Ruiz & Pav.) Kajian Pustaka Aspek Botani, Kandungan Kimia, dan Aktivitas Farmakologi
33

Analisis Kadar Merkuri dan Hidrokuinon dalam Kosmetik Krim Pemutih yang Dijual di Online Shop
29

Studi Farmakokinetika Teofilina Setelah Pemberian Oral Dosis Tunggal Tablet Teofilina dan Aminofilina Lepas Kendali pada Subyek Normal
27

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Research Article

Rhizome Buds Disinfection for Preparation of Red Ginger (*Zingiber officinale* Roxb. var. rubrum Rosc.) *In Vitro* Culture

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ABSTRACT: *The success of culture initiation depends on explant surface sterilization techniques. Suitable concentration, combinations, and duration of exposure of sterilizing agents are important to raise in vitro culture successfully. The aim of this work is to obtain the suitable sterilization method for explant buds of red ginger rhizome to get the axenic culture. Four sterilizing agents, fungicide, bactericide, Cefotaxime antibiotic, and NaOCl were tested for sterilization by various concentration and duration of exposure. The results showed that sterilizing agents 200 mg/L Cefotaxime and 100 mg/L Benomyl combined with NaOCl decreased the contamination of explants, and achieved 20% axenic culture.*

Keywords: *rhizome bud; red ginger; explant sterilization methods*

1. Introduction

Red ginger (*Zingiber officinale* Roxb. var. rubrum Rosc.) belonging to the family Zingiberaceae is one of the most important components of Indonesian herbal medicine, locally called as *jamu*. Red ginger is also one of the most important export commodities. High fluctuation price of Indonesian red ginger is caused by the lack of the market stock and serious damage due to pests and diseases [1]. The use of healthy planting material is necessary to be considered to increase red ginger productivity [2].

Plant tissue culture technique has been used for the purpose of *in vitro* propagation on various species. *In vitro* propagation aims to achieve healthy plants that will be used as seeds [3]. Therefore, we need the suitable sterilization process to eliminate microorganism contaminant in explants so as not to interfere plant growth.

The explants sterilization is the process of making explants contamination free before establishment of cultures. It is important that the explants be free of any contaminants including endophytic prior to tissue culture without losing their biological activity [4]. These factors such as source of explants, plant species, age and other climatic changes affect the success of sterilization methods [5]. The problem at the beginning of culture is contamination of microorganisms and browning in explants. Therefore, suitable sterilization techniques are needed to eliminate all microorganisms on the surface and inside explants so that they do not interfere and reduce the growth of explant cells.

Internal contaminants are very difficult to overcome because sterilization is only limited to the surface of explants. In plant material that contains inside contaminants, systemic antibiotics or fungicides must be used [6-9].

Various sterilizing agents are used to decontaminate plant tissues for *in vitro* cultures. The sterilizing agents are also toxic to the explants, so it is necessary for suitable concentration of sterilant, exposure duration of the explants to various sterilant, the sequences stage of using these sterilant have to be standardized to minimize explants injury and increase survival rate while ensuring effective sterilization [10].

According to Srivastava *et al.* [11], concentration and exposure duration of the sterilizing agents differ for different plant and plant parts. Tiwari *et al.* [12] reported that the use of only one sterilizing agent is not successful to sterilize the sugarcane leaf sheath. Sodium hypochlorite (NaOCl) combined with antibiotics is an effective sterilizing agent to eliminate bacterial contaminants in red durian leaf explants [13], shoot tip and leaf sheath explant of *Ensete ventricosum* [14]. Patel *et al*. [9] reported that the submersion of nodal explants of pomegranate cv. *Bhagva* in antibiotic solution effectively produce axenic culture that are able to grow. The focus of this study is to develop a standard protocol for the sterilization of rhizome bud from red ginger rhizomes to obtain axenic culture.

2. Materials and methods

2.1. Chemicals

Murashige-Skoog (MS) medium was used as a culture medium, gelled with 2.5 g/L Phytagel, and 3% sucrose as carbon source. HCl or NaOH adjusted pH of medium to 5.8. Bayclin (NaOCl 5.25%) and Cefotaxime antibiotic were used as sterilizing agent to eliminate the surface contaminant of explants. Fungicide (Dithane M-45) and bactericide (Benlate), Benomyl fungicide, Cefotaxime antibiotic were used as systemic sterilizing agent to remove the contaminant inside tissue of explant. Plant growth regulators were added to medium, i.e. benzyl aminopurine (BAP), naphthalene acetic acid (NAA), and gibberellic acid. Polyvinylpyrrolidone (PVP-40) was used as antioxidant to prevent browning at explants, and Tween-20 was used as surfactant to help the sterilizing agent penetrate in to tissue of explant.

2.2. Plant materials

The explants used were part of the bud

meristem of the red ginger rhizome from Yogyakarta. The red ginger rhizomes were washed thoroughly in running water for 30 minutes before being sterilized with fungicide and bactericide.

2.3. Method development

2.3.1. Explant surface sterilization

Rhizome bud explants were washed for 30 min with running tap water with liquid detergent. All thin leaves were removed from rhizome. Rhizome buds were then soaked in fungicide (2 g/L) for 120 min, washed three times by sterile distilled water, then treated with bactericide (2 g/L) (for 180, 360 min). Thereafter, the rhizome bud explants were treated by applying 6 sterilization methods (M1-M6) as presented in Table 1. In contrast to the sterilization methods (M7) without soaked in fungicide and bactericide, the rhizome bud explants were immediately soaked in antibiotic (Cefotaxime) and fungicide (Benomyl).

Shoot apex meristem (± 2.0 mm) were excised from buds after sterilizing treatment, and planted on each treatment media containing gibberellic acid or without gibberellic acid. The cultures were incubated with a photoperiod of 16 h/8 h light/dark, temperature 25±2 °C and 60 to 70% relative humidity.

2.3.2. Culture media

There were 2 treatments of plant growth regulator (0.5 mg/L gibberellic acid or without gibberellic acid) added to MS medium. MS medium supplemented with 3.0 mg/L BAP + 0.5 mg/L NAA + 200 mg/L PVP.

2.3.3. Data analysis

Experiments were organized according to completely randomized design with 20 explants per treatment. The cultures were maintained for 8 weeks. Culture had also been documented photographically with the use of stereoscopic

Table 1. Explants surface sterilization procedure

Treatment code	Sterilization procedure
M1	2 g/L fungicide (120 min), washed, 2 g/L bactericide (360 min), washed, NaOCl/sterile distilled water (1:1) plus 2 drops of Tween-20 for 5 min, washed, NaOCl/sterile distilled water (1:2) for 10 min, followed by five times washing using sterile distilled water.
M2	2 g/L fungicide (120 min), washed, 2 g/L bactericide (360 min), washed, NaOCl/sterile distilled water (1:1) plus 2 drops of Tween-20 for 10 min, washed, NaOCl/sterile distilled water (1:2) for 15 min, followed by five times washing using sterile distilled water.
M3	2 g/L fungicide (120 min), washed, 2 g/L bactericide (360 min), washed, NaOCl/sterile distilled water (1:1) plus 2 drops of Tween-20 for 15 min, washed, NaOCl/sterile distilled water (1:2) for 20 min, followed by five times washing using sterile distilled water.
M4	2 g/L fungicide (120 min), washed, 2 g/L bactericide (180 min), washed, 100 ppm Cefotaxime for 60 min, washed, NaOCl/sterile distilled water (1:1) plus 2 drops of Tween-20 for 10 min, washed, NaOCl/sterile distilled water (1:2) for 15 min, followed by five times washing using sterile distilled water.
M5	2 g/L fungicide (120 min), washed, 2 g/L bactericide (180 min), washed, 100 ppm Cefotaxime for 60 min, washed, NaOCl/sterile distilled water (1:1) plus 2 drops of Tween-20 for 5 min, washed, NaOCl/sterile distilled water (1:2) for 10 min, followed by five times washing using sterile distilled water.
M6	2 g/L fungicide (120 min), washed, 2 g/L bactericide (120 min), washed, 100 ppm Cefotaxime for 120 min, washed, NaOCl/sterile distilled water (1:2) plus 2 drops of Tween-20 for 5 min, washed, NaOCl/sterile distilled water (1:3) for 10 min, followed by five times washing using sterile distilled water.
M7	100 mg/L Benomyl for 60 min, washed, 200 mg/L Cefotaxime for 60 min, washed, NaOCl/sterile distilled water (1:2) plus 2 drops of Tween-20 for 5 min, followed by five times washing using sterile distilled water.

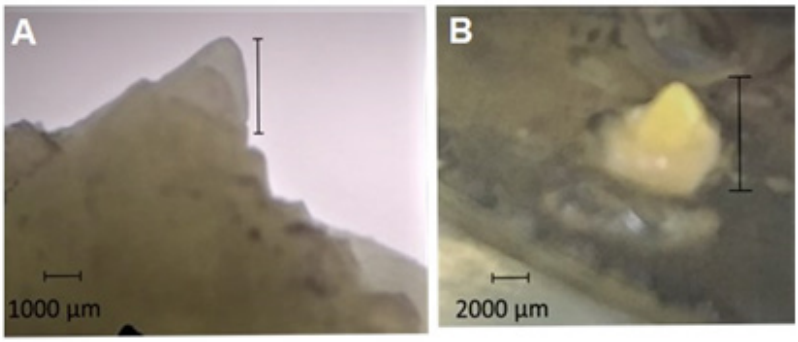

Figure 1. Explant shoot apex meristem from rhizome bud at early culture (A) and after 2 weeks of culture (B). A: shoot apex meristem of red ginger rhizome bud (in stereoscopic microscope); B: shoot apex meristem of rhizome bud after 2 weeks of culture

microscope. The percentage of contaminated explants and also the survived free contaminated were counted.

3. Results and discussion

3.1. Explant surface sterilization

After observing for one week of planting all surface sterilization treatment were almost contaminated. As shown in Figure 1A and B, there were no browning at the explants. The addition of 200 mg/L PVP to MS medium improved the recovery of isolated meristem and also prevented the browning. Similar results with PVP (300 mg/L) was observed in overcoming explant browning of young stem segments of Hongyang kiwifruit by Chai *et al.* [15].

Table 2 revealed the percentage of survived or contaminated explants. Bacterial and fungi contaminants caused mortality of explants (treatment code M1, M2, M3) which were also caused by too long exposure duration to sterilizing agents, so the tissue of explant became damage. High concentration and long exposure duration to sterilizing agents resulted contaminant free explants but loss of explants viability (treatment code M4, M5, M6). The contaminant within the explants is not easily controlled. Antibiotic with broad spectrum activity is prerequisite to obtain better results. Cefotaxime antibiotic is mainly used against bacteria attack in humans. However, it is also used to eliminate bacteria in plant genetic transformation and low toxicity to plants [16]. Higher concentration of 300 - 400 mg/L Cefotaxime could inhibit the growth and differentiation of plant tissue. Based on Table 2, the explants exposed to 100 mg/L Benomyl for 60 min, 200 mg/L Cefotaxime for 60 min, followed by NaOCl with lower concentration and shorter duration of treatment (M7 code) gave 20% axenic cultures and survived. Oo *et al.* [8] explained that strawberry explants which were treated with reduction time of exposure (20% NaOCl for 5 min, 70% ethanol for 5 min, 0.1% mercuric chloride HgCl2 for 4 min) and higher concentration of antibiotic (500 mg/L Ciprofloxacin for 60 min) gave the best results with higher survival percentages 25%, 10%, 55%, 20% respectively on the shoot tips, runner tips, nodal segments, and leaf segments. Patel *et al.* [9] stated that exposing nodal segments of *Punica granatum* L. to antibiotics 300 mg/L Bavistine for 18 min, 200 mg/L each of Streptocycline and Cefotaxime for 18 min and 8 min followed by 1000 mg/L HgCl2 for 5 min were achieved to be most the effective treatment to obtain 93.33% axenic culture. Mercuric chloride is often effectively used to overcome microbial contamination on underground rhizomes. The use of mercuric chloride in this work was avoided because it is

Table 2. Response of red ginger to 7 combinations of explant sterilization for 8 weeks of culture

Treatment code	Sterilization methods	Time (minute)	Initiated explants	Explants response (%)*			
				Browning	Survived, free contaminated	Not survived, free contaminated	Contaminated explants
M1	Fungicide	120	20	0	0	0	100
	Bactericide	360					
	NaOCl/distilled water (1:1)	5					
	NaOCl/distilled water (1:2)	10					
M2	Fungicide	120	20	0	0	0	100
	Bactericide	360					
	NaOCl/distilled water (1:1)	10					
	NaOCl/distilled water (1:2)	15					
M3	Fungicide	120	20	0	0	0	100
	Bactericide	360					
	NaOCl/distilled water (1:1)	15					
	NaOCl/distilled water (1:2)	20					
M4	Fungicide	120	20	0	0	70 (14/20)	30 (6/20)
	Bactericide	180					
	Cefotaxime 100 mg/L	60					
	NaOCl/distilled water (1:1)	10					
	NaOCl/distilled water (1:2)	15					
M5	Fungicide	120	20	0	0	80 (16/20)	20 (4/20)
	Bactericide	180					
	Cefotaxime 100 mg/L	60					
	NaOCl/distilled water (1:1)	5					
	NaOCl/distilled water (1:2)	10					
M6	Fungicide	120	20	0	5 (1/20)	75 (15/20)	20 (4/20)
	Bactericide	120					
	Cefotaxime 100 mg/L	120					
	NaOCl/distilled water (1:2)	5					
	NaOCl/distilled water (1:3)	10					
M7	Benomyl 100 mg/L	60	20	0	20 (4/20)	70 (14/20)	10 (2/20)
	Cefotaxime 200 mg/L	60					
	NaOCl/distilled water (1:2)	5					

*(…/…): ratio of number of observed result to total number of explants

the most toxic elements for organisms and ecosystem. Meanwhile many authors argued that the contaminant of the underground rhizomes was very high, so the establishment of contamination-free cultures was difficult, especially in this work with *Zingiber officinale* Roxb. var. rubrum Rosc. rhizomes.

Main contaminant at explants were bacteria that appeared after 3 days of culture. It is well known and most of the literature report NaOCl has broad spectrum of antimicrobial activity to rapidly kill bacteria, fungi, protozoa, and viruses.

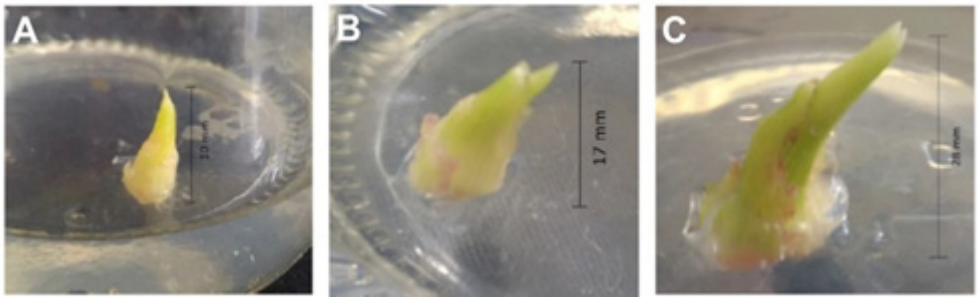

Figure 2. Development of in vitro shoots culture after 4 (A), 6 (B), and 8 (C) weeks of culture

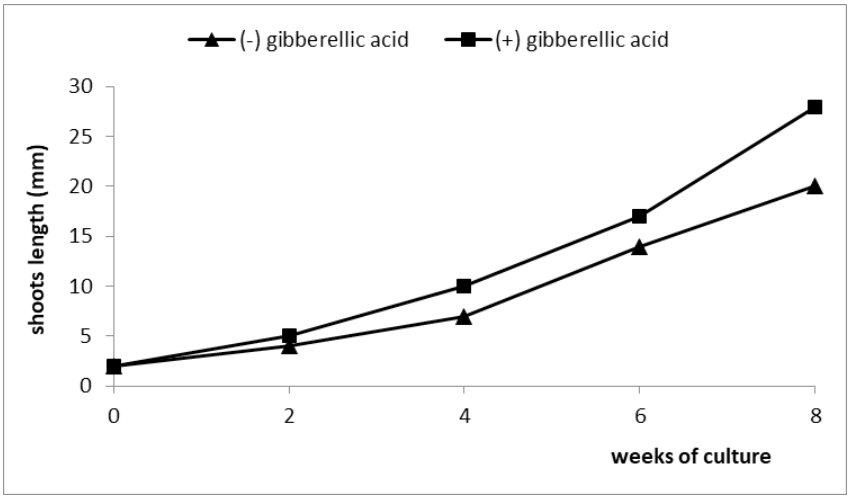

Figure 3. Graph of *in vitro* shoots length on MS medium supplemented by gibberellic acid or without gibberellic acid

For that reason, all treatment of sterilization methods in the present work uses NaOCl.

3.2. Growth of shoot apex meristem explant on MS medium

There were different developments of the culture on MS media containing gibberellic acid (Figure 2) or without gibberellic acid. Elongation of shoot apex meristem showed that the addition of gibberellic acid improved shoot elongation (Figure 3), nevertheless shoot multiplication had not yet been achieved, even though cultured in MS medium with the addition of BAP 3.0 mg/L. Gibbrellic acid has relatively broad spectrum in the form of cell enlargement. On the contrary, Zuraida *et al*. [17] reported that BAP concentration between 3.0-5.0 mg/L was very effective to promote ginger microshoots after 35 days resulted 2 microshoots per explant. BAP is commonly used to produce ginger multiple shoots [18, 19].

4. Conclusions

The best results with 20% axenic culture and alive were obtained using combinations of 200 mg/L Cefotaxime for 60 min, 100 mg/L Benomyl for 60 min, and NaOCl for 5 min.

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