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A novel explants disinfection method for Boesenbergia pandurata, an Asian medicinal plant, intended for in vitro culture initiation

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Abstract

Background. *Boesenbergia pandurata* (L.) Mansf. Kulturpf belongs to the Zingiberaceae family and is a medicinally valuable plant rich in flavanones, chalcones, and essential oils. These phytochemicals have been applied as spices in foods and used to traditionally cure diseases such as diarrhoea, dermatitis, cough, and mouth ulcer. Valuable biomass production of *B. pandurata* may be improved by culturing the plant *in vitro.* In order to introduce the species into *in vitro* culture, initial explants from donor plants must be effectively disinfected, applying an individually developed disinfection protocol, which is a complicated and often ineffective stage of initiation *in vitro* cultures. ***Aim of the study.*** The aim of the study was to develop an effective disinfection method of *B. pandurata* explants for establishing *in vitro* cultures of this medicinal plant. ***Material and methods.*** In this study, *B. pandurata* rhizomes were disinfected using (1) sodium hypochlorite (NaOCl) 70%, (2) mercuric chloride ($HgCl_2$) 0.1%, and (3) thermo-assisted ultrasonication in various temperature and time treatment using water and hydrogen peroxide solution. ***Result.*** Thermosonication improved the yield of sterile explants compared to the disinfection process using sodium hypochlorite and mercuric chloride. The best result is that 0% of contamination observed in explants occurred after disinfecting using ultrasonication at 50°C for 5 minutes using H_2O_2 as the medium. ***Conclusion.*** Thermosonication lowered the number of contaminations for *B. pandurata in vitro* culture initiation. To our best knowledge, this is the first report on the implementation of thermo-assisted ultrasonication to surface disinfect explants intended for *in vitro* cultures. *(Farm Współ 2023; 16: 3-11) doi: 10.53139/FW.20231602*

Keywords: thermosonication, surface sterilization, in vitro cultures, medicinal plants, fingerroot

Introduction

Among many medicinal plants in South-East Asian countries, one plant with many names has been regularly consumed to cure several diseases. It is the *Boesenbergia pandurata* Roxb. Schlecht or *Boesenbergia rotunda* (L.) Mansf. Kulturpf or *Kaempferia rotunda* Roxb., commonly known as temu kunci (Indonesia and Malaysia), krachai (Thailand), fingerroot, and Chinese keys (international name). The pharmacological value of *B. pandurata* exists thanks to the flavanones, flavones, chalcone, and essential oils composition in the rhizome. Fingerroot has been studied to have anti-inflammatory [1-4], antibacterial [5-8], anticancer [9-18], antioxidant [19-22], and other medical properties. As the demand for fingerroot has been increasing each year, conventional breeding has become inapplicable, and more effective and efficient methods are needed [23].

Surface disinfection is the essential step of *in vitro* culture initiation, as this method requires a contaminant-free condition. The surface sterilization process reduces or removes the surface contaminants but does not penetrate the plant tissue. Hence, endogenous microorganisms are often not affected. The most used chemical for explants' surface disinfection is sodium hypochlorite (NaClO) or other commercial bleaches. Less than 5% of the active chemical in these solutions

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is hypochlorous acid (HOCl), a potent oxidizing agent with antimicrobial activity [24]. Mercuric chloride ($HgCl_2$) is often used during surface disinfection treatment for highly contaminated explants. The concentration and exposure times vary depending on the type and size of initial explants [24]. $HgCl_2$ can be used as a sole sterilizing agent during the treatment or in combination with alcohol (e.g., in the disinfection process of *Stahlianthus thorelii* Gagnep [25]). Standard protocol of surface disinfection usually includes ethanol 70% treatment and NaClO 5-20% with surfactant addition (e.g., Tween-20, Tween-80). Many variables should be considered during this process, such as tissue type, source, and plant species. Optimizing the concentration of disinfection agents, combinations of agents, or explant's time exposure is highly recommended [26].

Ultrasonication (i.e., frequencies between 18-100 kHz) possesses antimicrobial effects and a valuable role in food preservation [27]. Ultrasonication could also preserve the products' nutrition composition/phytochemicals, texture, aroma, and flavour [28]. The bactericidal effect of ultrasound happened due to pressure changes that could disrupt the cellular structures of bacteria [29]. However, applying sonication alone is ineffective in inactivating microorganisms, especially spore-producing bacteria that are resistant and require hours of ultrasonication. Simultaneous application of ultrasound and temperature treatment (sublethal or lethal temperature) could improve microorganism inactivation and reduce the sterilization processes [29,30]. Higher temperatures would weaken the intermolecular force, thus enhancing the disruption by ultrasound [30]. Therefore, the food and beverage industries nowadays rely on the thermosonication method to prolong the shelving period and maintain the products' quality [28,31].

This paper tested and compared three surface disinfection methods (NaOCl, $HgCl_2$, and thermosonication) on fingerroot rhizome fragments for *in vitro* culture initiation. To our best knowledge, this is the first report on the thermo-assisted sonication method to establish *in vitro* culture.

Material and method

Plant material and media

The rhizomes of *Boesenbergia pandurata* Roxb. Schlecht were collected from a traditional market in Depok, West Java, Indonesia, in September 2021. The rhizomes were stored at 4°C until the time of the experiment (5 months). Mature rhizomes of fingerroot were chosen for the experiment.

Murashige and Skoog (MS) media consisted of sucrose 30 g/L, agar 7.2 g/L, and the pH then adjusted to 5.7-5.8 prior to autoclaving. The solidified MS media were used as *in vitro* media after surface disinfection from all tested methods. The surface disinfected explants were then put in a controlled room with a 16h/8h per day photoperiod and constant temperature of 20±1°C. The explants were observed three weeks post surface disinfection, and the contamination percentage is calculated based on the following equation (1):

$$\frac{number\ of\ explants\ contaminated\ by\ fungi/bacteria}{number\ of\ inital\ explants} \times 100\% \quad (1)$$

Disinfection method using sodium hypochlorite

Before performing surface disinfection in an aseptic condition, the rhizomes were first washed and brushed lightly under running water for 10 minutes. The rhizomes were then rewashed for 5 minutes in water with a few drops of Tween-20 (Sigma). After washing with detergent, the rhizomes were rinsed once in distilled water and brought into a sterile laminar airflow cabinet for further processing.

Inside the cabinet, the rhizomes were immersed in ethanol 70% for 30 seconds and surface disinfected in commercial bleach 70% v/v added with a few drops of Tween-80 (Sigma) for 15 minutes. Rinsing between steps and on the last step (3 times and 5 minutes each time) was performed with sterile distilled water. The fingerroot rhizomes were then cut into smaller fragments and put into solidified Murashige and Skoog (MS) media. Twenty explants were used in a single experiment, and the experiments were replicated two times. Thus, in total, 40 explants were used in this experiment.

Disinfection method using mercuric chloride

The pre-washing of fingerroot rhizomes was the same as the previously mentioned process. In an aseptic condition in a laminar airflow cabinet, the rhizomes were first submerged in ethanol 70% for 30 seconds and then rinsed once with distilled water. Mercuric chloride ($HgCl_2$, Chempur, PL) 0.1% was used at the end of the disinfection process to cover the rhizomes for 3 minutes. Finally, the rhizomes were rinsed three times with sterile distilled water for 5 minutes each. The fingerroot rhizomes were then fragmented and placed in solidified MS medium. Thirty explants were used in

a single experiment and replicated twice. Therefore, 60 explants were used in the experiment.

Disinfection method using thermosonication (water and hydrogen peroxide)

In this study, two treatments were used and analyzed to see the decontamination effect of thermos-assisted ultrasonication on fingerroot rhizome. Three various temperatures were used (30°C, 40°C, and 50°C), and the rhizomes were treated in two types of time treatment (5 minutes and 10 minutes). The first thermosonification process was completed using sterile distilled water as the medium. The second process was completed using hydrogen peroxide (H_2O_2) with the same temperature and time treatment variants.

The machine for ultrasonication with a heating feature (POLSONIC, Polsonic Palczynski Sp. J., type SONIC-3, PL) was cleaned with 70% ethanol, put into the laminar airflow cabinet, and sterilized under UV light. In the meantime, fingerroot rhizomes were pre-washed identically to the methods described earlier. Inside the cabinet, the explants were immersed in ethanol 70% for 30 seconds and then rinsed with distilled water prior to the thermosonication process. The POLSONIC ultrasonication machine was running on 40 kHz frequency, 2x160 Watts power, and on selected temperatures (30°C, 40°C, and 50°C) for 5 minutes or 10 minutes, respectively. The fingerroot rhizomes were then fragmented and placed in solidified MS medium; ten explants were obtained for each treatment.

Microscopic observation of the contaminants

Contaminants were observed under Leica DM750 RH microscope using 40-100x magnification. Figures were captured using Leica Application Suite version 3.1.0 (Leica Microsystems Limited, CH).

Result

One of the biggest obstacles to the effectiveness of plant tissue culture is the contamination of *in vitro* cultures from diverse sources. In order to obtain initial aseptic explants for *in vitro* experiments, surface disinfection (or surface sterilization) is an essential precondition. In this study, we evaluated the efficiency of three different methods: chemical disinfection using sodium hypochlorite (NaOCl), mercuric chloride ($HgCl_2$), and thermos-assisted ultrasonication using water and hydrogen peroxide (H_2O_2) on *Boesenbergia pandurata* rhizome fragments.

Sodium hypochlorite (NaOCl) is a potent oxidizing agent. Therefore, it is commonly used to sterilize tissue culture explants. In this work, however, the surface disinfection of fingerroot rhizomes with NaOCl yielded disappointing results since all explants were contaminated with fungi or/and bacteria (Table 1). Additionally, several other amounts of sodium hypochlorite were tested, and the outcomes were comparable in each case (data now shown). Nevertheless, no browning explants were observed in this study. Surface disinfection of the fingerroot rhizomes with mercuric chloride ($HgCl_2$) improved the contamination rate (Table II). The fungal contaminants produced by the explants were significantly decreased by around 35-55% compared to the disinfection process using NaOCl. However, necrotic fragments were seen in the tissues following exposure to the potent sterilant, negatively impacting the fingerroot explants. Several contaminants that occurred from both experiments were observed macroscopically and microscopically, as shown in Table V and Figure 1.

Three contaminants were identified based on macroscopic and microscopic observation (Figure 1). Contaminant A had grey and raised filamentous sporangia with a cottony appearance (Table VA). The

Table I. Contamination occurring after the surface disinfection process using NaOCl

	Number of explants	Contamination percentage	Type of contaminations
Replication 1	20	100%	Fungal contamination
Replication 2	20	100%	Fungal and bacterial contamination

Table II. Contaminants occurring after the surface disinfection process using $HgCl_2$ 0.1%

	Number of explants	Contamination percentage	Type of contaminations
Replication 1	30	43.3%	Fungal and bacterial contamination
Replication 2	30	63.3%	Fungal and bacterial contamination

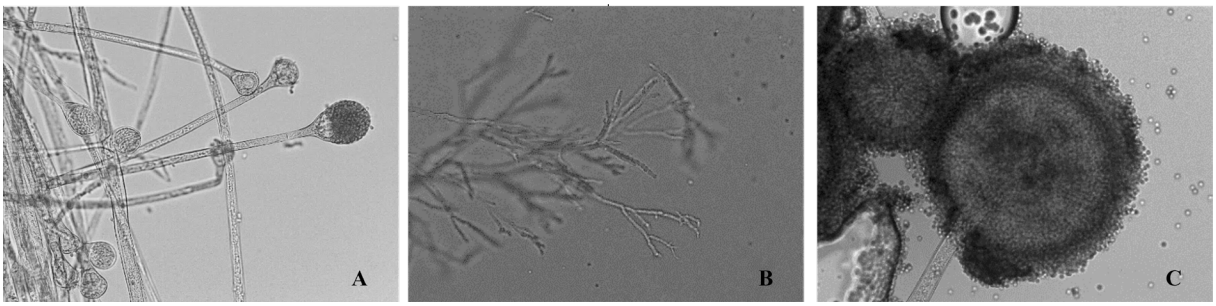


Figure 1. Microscopic observation of several contaminants. Probable *Rhizopus* sp. (A), *Streptomyces* sp. (B), and *Aspergillus* sp. (C) in 40x lens magnification [12.5 x 1.5 x 40]

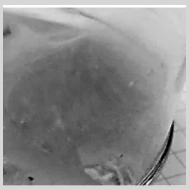
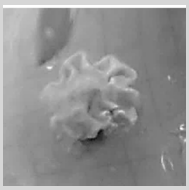
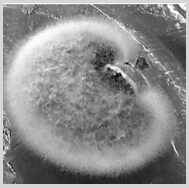
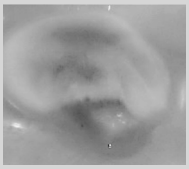
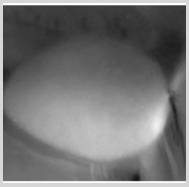
Table III. Contaminants occurring after the disinfection process using thermal-assisted ultrasonication with water medium

	Temperature	Time treatment	Number of explants	Contamination percentage	Type of contaminations
Replication 1	30°C	5 minutes	10	80%	20% bacterial contamination 60% fungal contamination
		10 minutes	10	100%	Fungal contamination
	40°C	5 minutes	10	80%	Bacterial contamination
		10 minutes	10	100%	Bacterial contamination
	50°C	5 minutes	10	100%	Bacterial contamination
		10 minutes	10	100%	Bacterial and fungal contamination
Replication 2	30°C	5 minutes	10	100%	Fungal contamination
		10 minutes	10	100%	Fungal contamination
	40°C	5 minutes	10	80%	60% bacterial contamination 20% fungal contamination
		10 minutes	10	100%	Bacterial contamination
	50°C	5 minutes	10	100%	Bacterial contamination
		10 minutes	10	100%	Bacterial and fungal contamination

Table IV. Contaminants occurring after the surface disinfection process using thermal-assisted ultrasonication with H_2O_2 medium

Temperature	Time treatment	Number of explants	Contamination percentage	Type of contaminations
30°C	3 minutes	10	10%	Bacterial contamination
	5 minutes	10	0%	-
40°C	3 minutes	10	70%	Bacterial contamination
	5 minutes	10	0%	-
50°C	3 minutes	10	50%	Bacterial contamination
	5 minutes	10	0%	-

Table V. Macroscopic observation of contaminants that occurred on fingerroot explants from non-thermosonication surface disinfection methods

Contaminants	Macroscopic observation				Image
	Form	Elevation	Margin	Color	
A	Filamentous	Raised	Filiform	Gray	
B	Circular	Umbonate	Undulate	White	
C	Filamentous	Raised	Filiform	Yellow, black, and white	
D	Circular	Raised	Filiform	Green and white	
E	Filamentous	Raised	Filiform	White	
F	Fillamentous	Raised	Filiform	White and black	

microscope's observation helped identify the sporangiophores, sporangium, and columella (Figure 1A). Thus, we concluded that the contaminant belongs to the Zygomycetes, *Rhizopus* sp. Contaminant B had white and circular with umbonate elevation and undulated margin (Table VB). Using 40x magnification, the con-

taminant had filamentous with extensive branching. The hypothesis for this contaminant from both observations was *Streptomyces* sp. from Actinomycetes. The third contaminant (Table VF; Figure 1C) had the characteristics of *Aspergillus* sp. The microscopic visualization showed the conidiophores and conidia head. These microorganisms are commonly found in soil.

This study is the first attempt to use the thermal-assisted ultrasonication method to surface disinfected fingerroot explants for *in vitro* purposes. Based on the results, using sterilized water as a medium for thermosonication was insufficient to obtain contaminant-free explants. As shown in Table III, all temperature and time-treatment variations had a significant contamination rate. However, bacteria contamination took precedence over fungal infection. In all temperature variants, better results were obtained on thermosonication using H_2O_2 as the medium in five minutes (Table IV). These results showed that using H_2O_2 as a medium improved the fungicidal and sporicidal activities in the thermos-assisted sonication method, compared disinfection process using sodium hypochlorite and mercuric chloride.

Discussion

The production of *B. pandurata* biomass or other medicinally valuable plants may be increased through *in vitro* culture of the plant. Initial explants from donor plants must be thoroughly cleaned using a developed disinfection protocol. Nevertheless, these methods are difficult to use and ineffective for introducing into *in vitro* culture. Park et al. (2021) observed that NaOCl was insufficient in controlling the contamination of the *Kaempferia parviflora* Wall. Ex Baker cultures [32]. A dual sterilization step using NaOCl was also used by Seranath et al. (2017) to sterilize *K. galanga* Linn explants prior to *in vitro* culture [33]. According to Afendi et al. (2013), utilizing sodium hypochlorite for surface disinfection in a single operation and doing it twice yields a superior outcome [34]. They tried six rhizomes' surface sterilization methods using various combinations and concentrations of NaClO, Tween-20, and ethanol. They concluded that pre-dipping the explant into a 95% ethanol solution, immersing in 20% (20 minutes) and 80% (10 minutes) NaOCl solutions with Tween-20, and dipping into 70% ethanol in between showed the minimum contamination and visible shoot response [34]. Wong et al. (2013) enforced vigorous stirring using a magnetic stirrer when the fingerroot buds were immersed in 20% v/v NaOCl for 20 minutes to improve the surface sterilization process [35].

Surface disinfection with $HgCl_2$ has been demonstrated to minimize the contamination in *B. pandurata* rhizomes [23,36,37], as well as in other Zingiberaceae, such as *K. angustifolia* [38], *Alpinia conchigera, A. galanga, Curcuma domestica, C. zedoaria, K. galanga* [39] and *C. kwangsiensis* [40]. Establishing an aseptic fingerroot culture without using mercuric chloride ($HgCl_2$) is challenging and consumes much time. Park et al. (2021) reported that in their preliminary results, the surface disinfecting *K. parviflora* explants using $HgCl_2$ yielded 65% sterile cultures, yet it also negatively affected the organogenesis and viability of explants [32].

A combination of NaOCl and 0.5% $HgCl_2$ as surface disinfecting agents for *B. pandurata* explants were used by Tan et al. (2003), Yusuf et al. (2011), and Yusuf et al. (2011a) [23,36,37]. The same approach was also used in numerous *in vitro* protocols of the Zingiberaceae family, such as *Curcuma kwangsiensis* [40], *Curcuma longa* [41], and *Kaempferia angustifolia* [38]. Unfortunately, this approach severely harms the environment with mercury poisoning.

Results on the thermosonication method that we obtained reflected a similar conclusion to the study by Baldry [42] that hydrogen peroxide is a potent sporicidal yet has poor bactericidal activity. It is possible that the H_2O_2 only cellularly injured the bacteria to some extent that it is repairable. Swartling and Lindgren (1968) studied the early H_2O_2 sporicidal effect on the spore-forming bacteria *Bacillus subtilis* [43]. From their study, increasing both H_2O_2 concentration and temperature significantly increased sporicidal properties. However, a thorough investigation will be needed to study the impact of high concentrations of H_2O_2 and high temperatures as surface disinfection protocol on explants intended for *in vitro* culture.

As previously mentioned, thermosonication has been widely used as a mechanic sterilant in the food and beverage industries. Nicolau-Lapeña et al. (2022) studied the effect of combined and non-combined ultrasound with temperature in strawberry disinfection technology [44]. Their study indicated a 3.8 log reduction in the inoculated *Listeria innocua* in strawberries with sonication 130 kHz and 55°C for 15 minutes. Furthermore, they observed that the combination treatment did not affect food quality or biochemical indicators except for minor colour and

texture alterations. Based on these findings, thermosonication seems to be a suiTable approach for surface disinfecting plant explants for *in vitro* cultivation. As a result, more research will be conducted to see if this approach influences secondary metabolite synthesis in *in vitro* plants.

Conclusion

Surface-disinfecting *Boesenbergia pandurata* rhizomes have been a challenge. Using either sodium hypochlorite or mercuric chloride was insufficient to obtain sterile explants for *in vitro* cultures. Thermo-assisted ultrasonication using sterile water to surface--disinfect the fingerroot explants was also insufficient. However, when H_2O_2 was used as the solution media in the thermosonication process, the number of sterile explants was significantly increased. Further optimization on surface disinfection of plant explants using the thermo-assisted sonication method is needed, for instance, by optimizing the concentration of hydrogen peroxide, optimizing the time and temperature treatment, or using peracetic acid as a medium to improve the bactericidal activities.

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Conflict of interest
None

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