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Effectiveness of Sterilization Methods of Coffee Leaf Explants (*Coffea canephora* Var. Milo Pace) To Decrease Contamination and Browning In Vitro

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ABSTRACT

Coffee is one of the leading plantation commodities that are in demand by the public. Robusta coffee var. Milo Pace is Jember's flagship local coffee that was only released at the end of 2023. Seedling propagation continues to be pursued to preserve the coffee. In vitro culture is one of the effective plant propagation methods for production in large quantities. However, explant sterilization is a crucial stage in in vitro culture. Reports related to in vitro culture of robusta milo pace have never been reported before. Therefore, optimization of sterilization methods is the initial stage in supporting the success of in vitro culture of robusta milo pace coffee. The purpose of this study was to obtain the optimal method of sterilization of leaf explants of robusta milo pace coffee. This study used a one-factor randomized design, namely the method of sterilization of coffee leaf explants consisting of 12 methods. The sterilants used were detergent, bactericide, fungicide, NaOCl, H_2O_2, alcohol and distilled water with different time and concentration. The results showed that sterilization of explants by method XII with 2 g/L detergent for 20 minutes, 2 g/L bactericide and 2 g/L fungicide for 60 minutes, 1.05% NaOCl for 15 minutes, 0.525% NaOCl 10 minutes and 1.5% H2O2 can suppress 20% contamination such as fungi and bacteria with an average contaminant appearance time of 11 days after inoculation. The percentage of browning was 20% with an average appearance of 8.8 days after inoculation. This method maintains the percentage of live explants up to 60% characterized by fresh green explants.

INTRODUCTION

Coffee (*Coffea canephora*) is one of the plantation commodities in demand in the global market. East Java ranks fifth as a coffee producer in Indonesia (Harum, 2022). Moreover, Jember is one of the fifth largest coffee-producing districts at the provincial level. Coffee cultivation in Jember is spread from the Argopuro mountains to Raung mountain consisting of arabica and robusta coffee (Purwandhini et al., 2023). This area has a local coffee, robusta milo

pace variety, which is cultivated in Pace village, Silo, Jember. However, the existence of the plant is threatened with extinction due to mass logging in 2024 (Kumparannews, 2023). Therefore, it is necessary to propagate the plants to preserve the local coffee varieties by utilizing limited planting materials.

In vitro culture is a solution in saving germplasm, rare plants and difficult to cultivate conventionally. This method is a plant propagation carried out in a bottle under controlled and aseptic environmental conditions (Handayani et al., 2022). Explants as planting material can come from all parts of the plant such as cells, leaves, stems, shoots, seeds or flowers. In addition, in vitro culture can produce a large number of seedlings in a relatively short time compared to field propagation. An important initial stage in in vitro culture is explant sterilization (Irsyadi, 2021; Suratman & Mulyani, 2013). This stage is the key to successful in vitro culture without bacterial and fungal contamination and explant browning (Handayani et al., 2021). Commonly used sterilants include detergents, bactericides, fungicides, sodium hypochlorite, hydrogen peroxide, alcohol and other chemicals that are anti-microbial (Felek et al., 2015). Different concentration levels, different soaking durations and explant types give different responses at the explant sterilization stage.

In vitro culture of coffee plants has been widely reported before. However, there have been no reports related to in vitro culture of robusta var. milo pace. The results by Asmono et al. (2021) showed that the sterilization method using liquid detergent 2 g / L, bactericide and fungicide 2 g / L, Erytomycin 4 g / L, 70% alcohol, NaOCl with a duration of 10 min can reduce the occurrence of contamination and the lowest browning in leaf explants of Arabica coffee var. andungsari and Robusta BP308. This is in line with Solin & Siregar, (2025) that the used of these sterilizers with different concentrations and durations can also suppress contamination and browning in gambier leaf explants (*Uncaria gambir* (Hunter) Roxb). Each different type of plant will give a different response to sterilant material. This study aims to obtain the optimal sterilization method on leaf explants of robusta coffee var. milo pace in vitro.

METHODS

Location and Time

This research was conducted in January - February 2025 at the Botany and Ecophysiology Laboratory, Faculty of Agriculture, Universitas Jember, Bondowoso campus. The explants used were young leaves of robusta coffee var. milo pace, Murashige and Skoog media, agar, sucrose, distilled water, detergent, 70% alcohol, bactericide, fungsida, NaOCl and aluminum foil. Equipment used such as autoclave, laminar air flow, dissecting kit, glassware, bunsen and UV.

Method

This study was designed using a single-factor Completely Randomized Design (CRD), namely sterilization method consisting of 12 treatment methods (Table 1). Each treatment was repeated 5 times with 3 samples per replicate. The stages of sterilization treatment of coffee leaf explants were presented in **Table 1.**

Table 1. Sterilization method robusta milo pace coffee leaf explants

No	Treatments
I	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 5,25% NaOCl for 10 minutes.
II	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungisida for 30 minutes > 1,05% NaOCl for 10 minutes > 0,525% NaOCl for 5 minutes > 70% alkohol for 30 sec.
III	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 3% H_2O_2 for 10 sec.
IV	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 1,5% H2O2 for 10 minutes > 70%Alkohol for 30 sec.
V	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 0,525% NaOCl for 10 minutes > 1,5% H_2O_2 for 10 minutes
VI	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 100 ppm Tween20 for 10 minutes > 1,05% NaOCl for 5 menit > 1,5% H2O2 for 5 minutes
VII	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 70%Alkohol for 30 sec > 0,525% NaOCl for 10 minutes > 1,05% NaOCl for 10 minutes
VIII	2 g/L detergent for 10 minutes > 2 g/L bakteriside and 2 g/L fungiside for 30 minutes > 5,25% NaOCl for 10 minutes > 0,525% NaOCl for 15 minutes
IX	2 g/L detergent for 15 minutes > 2 g/L bakteriside and 2 g/L fungiside for 45 minutes > 1,05% NaOCl for 10 minutes > 3% H2O2 for 10 minutes > 70% alkohol for 3 sec
X	2 g/L detergent for 15 minutes > 2 g/L bakteriside and 2 g/L fungiside for 60 minutes > 5,25% NaOCl for 15 menit > 1,05% NaOCl for 10 minutes > 70% alkohol for 30 sec
XI	2 g/L detergent for 15 minutes > 2 g/L bakteriside and 2 g/L fungiside for 45 minutes > 5,25% NaOCl for 15 minutes > 1,05% NaOCl for 10 minutes > 0,525% NaOCl for 5 minutes
XII	2 g/L detergent for 20 minutes > 2 g/L bakteriside and 2 g/L fungiside for 60 minutes > 1,05% NaOCl for 15 minutes > 0,525% NaOCl 10 minutes > 1,5% H_2O_2

Explants Inoculation

Sterilization of young robusta milo pace coffee leaves is done by washing the leaves under running water for 5 minutes to clean the dirt that is still attached to the surface of the explants. Next, the explants were sterilized according to the method used (Table 1). Each time through the soaking stage, the explants were rinsed using distilled water 3 times. Explants were then cut in antiseptic solution, then inoculated on Murashige and Skoog media. The incubation period of the explants was carried out for 4 weeks in a bright incubation room at room temperature of 23±2°C.

Data Analysis

The data consisted of percentage and time of appearance of contamination and browning, type of contamination and live explants. The quantitative data obtained were analyzed using ANOVA, if there was a significant difference, the DMRT further test was carried out with a confidence level of 95%.While qualitative data were analyzed descriptively.

RESULTS AND DISCUSSIONS

Effect of Sterilization Method On Contamination Explant

Based on the study (**Table 2**), it was known that sterilization methods X and XI were the best treatments in suppressing explant contamination. It was obtained that the explants of robusta milo pace coffee leaves did not appeare contamination. While the other treatments experienced explant contamination reaching 100%. The initial time of explant contamination appeared for the first time in each treatment occurred starting on the fourth day to the eleventh day after planting. In addition, the types of contaminants that appeared were dominated by fungi in each treatment. In the treatment of sterilization methods II, VII, VIII and XII, bacteria and fungi appeared. The used of 2 g/L detergent solution for 15 minutes, 2 g/L bactericide and fungicide solution for 60 minutes, 5.25% NaOCl solution for 15 minutes, 1.05% NaOCl for 10 minutes and 70% alcohol for 30 seconds has been effective in suppressing contamination of robusta milo pace coffee leaf explants.

Table 2. Effect of sterilization method of robusta milo pace coffee leaf explants on the percentage of contaminated explants, the time to appear contaminants and their types at the age of 4 weeks after planting.

Sterilization Method	Percentage of Contamination (%)	Time Appeare Contamination (DAI)	Contaminant
I	100[a]	5	Fungi
II	100[a]	3,6	Fungi and bacteria
III	100[a]	4	Fungi
IV	100[a]	4	Fungi
V	100[a]	4	Fungi
VI	100[a]	5,6	Fungi
VII	100[a]	7,4	Fungi and bacteria
VIII	100[a]	9	Fungi and bacteria
IX	100[a]	5,8	Fungi
X	0[b]	-	-
XI	0[b]	-	-
XII	20[b]	11	Fungi and bacteria

Note: numbers followed by the same letter indicate that it is not significantly different from the percentage of browning explants in the DMRT test α 5%.

Various sterilants can reduce the occurrence of explant contamination, which is influenced by the concentration and soaking time. Detergent, bactericide and fungicide solutions are commonly used as basic sterilization. In addition, NaOCl and H2O2 are used as advanced sterilants carried out in laminar air flow (Etty Handayani et al., 2021). NaOCl is classified as a hypertonic disinfectant that can cause hydrolysis of ormosis in microbial cells. Moreover, NaOCl has a high degree of actively controlling microbes (Singh et al., 2011). This active

ingredient is widely contained in commercial clothes bleaching solutions that can be used directly.

Felek et al., (2015) reported that sterilization using 0.5% NaOCl for 20 minutes was effective for suppressing contamination, but obtained the highest explant mortality rate of 71.43% in peach (*Prunus persica* L.) node explants. Setiani et al., (2018) reported that sterilization of breadfruit (Artocarpus altilis) leaf explants using 5.25% NaOCl for 10 minutes was effective in suppressing contamination, but resulted in explant browning reaching 90%. Natasha & Restiani, (2019) reported that pre-sterilization with 10% detergent followed by 70% alcohol for 30 seconds and chlorox for 10 minutes effectively suppressed contamination in Java ginseng (*Talium paniculatum*) leaf explants.

Fig 1. Condition of coffee leaf explants a) coffee leaf explants contaminated with bacteria, b) coffee leaf explants contaminated with fungi, c) browning coffee leaf explants, d) green live coffee leaf explants

Fungi are the most common contaminants in explants. Fungal growth is characterized by the appearance of hyphae on the surface to cover the surface of the white to grayish explants. While bacterial contamination is characterized by the appearance of white to reddish-reddish mucus around the explants (Shofiyani & Damajanti, 2017). The time of appearance of contaminants generally occurs on the fifth day after inoculation. The contaminants are thought to come from microbes that remain on the surface of the explants that are not dissolved during sterilization. This is in accordance with research (Asmono et al., (2021) that the appearance of fungi begins on the fifth day after inoculation in robusta and arabica coffee leaf explants. Meanwhile, the appearance of contaminants more than 10 days after inoculation is thought to come from endogenous microbes that have entered the explant tissue (Handayani et al., 2022).

Effect of Sterilization Method On Explant Browning

Based on the analysis, the sterilization method had a significant effect on the percentage of browning. **Table 3** showed that sterilization method XI obtained the highest percentage of browning reaching 73.33% with an average browning time of 11 days after inoculation. This was followed by the X sterilization method treatment with a browning percentage of 46.66% with an average browning time of 9.2 days after inoculation. While the other treatments did not appiare browning because the explants had experienced contamination, so that the development of explants was inhibited. Explant browning was indicated by changes in the color of the explants from green to brownish on the surface of the explants (**Fig 1c**).

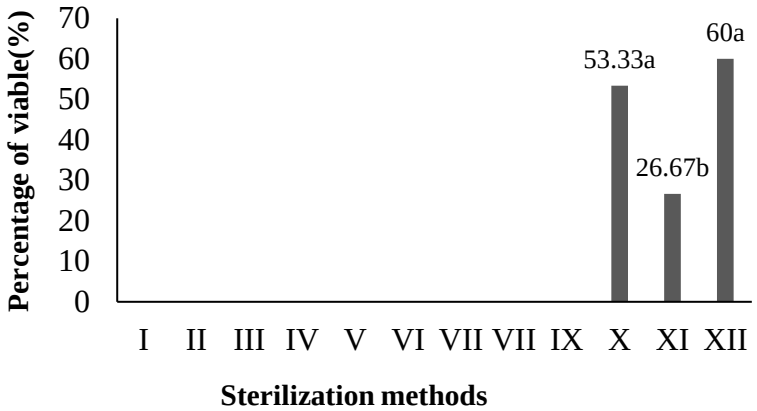
Table 3. Effect of sterilization method of robusta milo pace coffee leaf explants on the percentage of explant browning and time to appear browning

Sterilization Method	Percentage of Browning (%)	Time Appeare of Browning (DAI)
I	13,33[a]	2,8
II	0[c]	-
III	0[c]	-
IV	0[c]	-
V	0[c]	-
VI	0[c]	-
VII	26,66[c]	4,8
VIII	0[c]	-
IX	0[c]	-
X	46,66[b]	9,2
XI	73,33[a]	11
XII	20[c]	8,8

Note: numbers followed by the same letter indicate that it is not significantly different from the percentage of browning explants in the DMRT test α 5%.

The use of high concentrations of sterilants with a long duration can cause tissue damage and even death. This will have an impact on changing the color of the explants to browning. Browning is one of the problems that indicates the process of physiological deterioration of explants until they die in in vitro culture (Wulandari & Nasution, 2014). According to Handayani et al. (2021), explant browning is caused by phenolic compounds that come out of damaged tissues which are then oxidized and change the surface color to brownish. Setiani et al. (2018) added that immersion of explants in 5.25% NaOCl and 70% alcohol for 10 minutes causes permanent and irreversible tissue destruction.

Percentage of Viabel



Gambar 2. Percentage of viable based on DMRT α = 5%

Based on the results (**Fig 2**) showed that the best percentage of living explants was obtained in sterilization methods X and XII 53.33% and 60%, respectively. Living explants were characterized by the color of leaf explants remaining green and experiencing development such as imbibition which will then growed callus (Fig 1d). in addition to removing contamination, the optimal sterilization method can maintain the condition of the explants remain fresh without browning. Concentrations of 5.25% NaOCl and 1.5% H2O2 are commonly used in the sterilization process of in vitro cultured plant explants. However, soaking the explants for too long can cause explant damage (Rismayani & Hamzah, 2010; Setiani et al., 2018).

CONCLUSIONS

Sterilization explants by method XII with 2 g/L detergent for 20 minutes, 2 g/L bactericide and 2 g/L fungicide for 60 minutes, 1.05% NaOCl for 15 minutes, 0.525% NaOCl 10 minutes and 1.5% H_2O_2 can suppress 20% contamination such as fungi and bacteria with an average contaminant appearance time of 11 days after inoculatio. The percentage of browning was 20% with an average appearance of 8.8 days after inoculation. This method maintains the percentage of live explants up to 60% characterized by fresh green explants..

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