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Optimization of the sterilization method for leaf explant Robusta BP 308 coffee in vitro

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Abstract. The research aimed to know the optimal sterilizing agent for the success of the coffee callus inoculation from young leaf explant. This research was conducted at the Plant Tissue Culture Laboratory for 2 months. The explants are used from young leaves of Robusta clones BP 308. This experiment used a nonfactorial completely randomized design with three sterilization methods and was repeated 10 times. The treatments tested were as follows Method 1: Fungicide 2 gr/L, Bactericide 2 gr/L, Erythromycin 4 gr/L (20 min); 70% alcohol (3 sec); NaOCl 0.525% (10 min); NaOCl 1.05% (10 min). Method 2: 70% alcohol (3 sec), 0.1% HgCL2 (5 min). Method 3: Fungicide 2 gr/L, Bactericide 2 gr/L, Erythromycin 4 gr/L (20 min); 70% alcohol (3 sec); NaOCl 0.525% (10 min); NaOCl 1.05% (10 min); 0.1% HgCL2 (5 min). The results showed that the sterilization method used suppressed the rate of bacterial and fungal contamination, but there was no significant difference between the methods. A very significant effect was seen in the percentage of browning explants. In sterilizing agent treatment 1, there was no browning explant, while the other methods caused browning to reach 62.5%. Browning also causes differences in callus regeneration ability. The explants which were sterilized in method 1 showed a high percentage of callus (85%) and were also appear to callus faster at 11 days after inoculation.

1. Introduction

Robusta coffee clone BP 308 is a coffee genotype that has been confirmed as a superior planting material to have a high level of resistance to *P. Coffeae* nematode attack [1]. In its propagation, the type of robusta coffee is a cross-pollinated plant so it requires other types of robusta to produce seeds. Of course, it will affect the genetic uniformity of the seeds produced. One of the propagation methods that can produce genetically uniform seeds is through in vitro culture techniques. This technique is a method of plant propagation by isolating body parts or organs (cells or tissues) from plants and growing under sterile or aseptic conditions so that they can grow into whole plants. The advantage of this technique is that it produces plants that have the same or identical characteristics as the parent, producing large numbers of seeds in a relatively short time [2], [3].

In vitro culture on coffee, plants are usually used to produce Somatic Embryogenesis (SE) seeds [4], [5]. Cells from 1 piece of leaf explant measuring 1cm^2, were able to develop into hundreds of somatic embryos, each of which would later become seedlings with identical properties to the parent. However, the problem in the early stages of inoculation is the contamination of the explants. Contamination can inhibit the growth process so that the explants cannot grow or die. The problem arises because the inoculated explants are not sterile.

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The solution to suppress contamination is to use an appropriate sterilization method. Sterilization of explants aims to prevent or kill microorganisms that may be carried or attached to the leaves when explants are taken. There are several sterilization methods used by previous researchers, namely by immersion using a solution of the Dithane fungicide and Agrimycine bactericide, dipping in 50% alcohol and soaking in 5.25% NaOCl solution [6]. Then in another study there were washing explants with liquid detergent, soaked in a solution of 2 g/L Dithane fungicides, immersed in 70% alcohol, and soaked in NaOCl solution [7]. In its application, the sterilization method from the research conducted has not optimally obtained sterile explants, especially if there are different types of explants and the origin of the explants. The novelty in this study is that the researchers will use Robusta clones BP 308. Therefore, the authors want to conduct research to test and optimize several methods of sterilizing coffee leaf explants, to obtain a suitable sterilization method for the success of Arabica and Robusta coffee explant cultures in vitro, especially the origin of coffee leaf explants from the Jember-Indonesia.

2. Methods

2.1Preparation of plant material

The research was carried out for 2 month at the Culture Laboratory, Politeknik Negeri Jember, Indonesia. The explants used were young leaves from coffee seedlings aged 4-5 months.

2.2 Surface sterilization

Explants in the form of young leaves were cut and sterilized using 3 treatments of sterilization methods, as shown in **Table 1** below:

Table 1. Sterilization methods of coffee leaf explants

Treatment	Sterilizing agent	Concentration	Soaking time
Sterilization Method 1	Liquid detergent	2 ml/L	10 minutes
	Rinse sterile distilled water 3 times		1 minute
	Dithane Fungicide	2 gr/L	20 minutes
	Agrimycine Bactericide	2 gr/L	20 minutes
	Erythromycin	4 gr/L	20 minutes
	Rinse sterile distilled water 3 times		1 minute
	Alcohol	70%	3 seconds
	Rinse sterile distilled water 3 times		1 minute
	NaOCl (5.25%)	10%	10 minutes
	NaOCl (5.25%) 20%	20%	10 minutes
	Rinse sterile distilled water 3 times		1 minute
Sterilization Method 2	Liquid detergent	2 ml/L	10 minutes
	Rinse sterile distilled water 3 times		1 minute
	Alcohol	70%	3 seconds
	Rinse sterile distilled water 3 times		1 minute
	$HgCL_2$	0,1%	5 minutes
	Rinse sterile distilled water 3 times		1 minute
Sterilization Method 3	Liquid detergent	2 ml/L	10 minutes
	Rinse sterile distilled water 3 times		1 minute
	Dithane Fungicide	2 gr/L	20 minutes
	Agrimycine Bactericide	2 gr/L	20 minutes
	Erythromycin	4 gr/L	20 minutes
	Rinse sterile distilled water 3 times		1 minute
	Alcohol	70%	3 seconds
	Rinse sterile distilled water 3 times		1 minute
	NaOCl (5.25%)	10%	10 minutes

	NaOCl (5.25%) 20%	20%	10 minutes
	Rinse sterile distilled water 3 times		1 minute
	$HgCL_2$	0,1%	5 minutes
	Rinse sterile distilled water 3 times		1 minute

2.3Culture medium
Basal medium for callus induction using 1/2 MS medium [8] with 1ppm 2,4-D dan 2ppm Kinetin. MS medium was adjusted at pH 5.8, then autoclaved at 121 psi pressure for 30 minutes.

2.4Explant inoculation
Leaf explants were taken from the parent plant, then sterilized according to the tested method (**Table 1**). In Laminar Air Flow, leaf explants were cut into squares of 1 cm^2, then implanted in culture media and incubated in the dark. Each bottle contains 1 explant. Observations are made every day to find out if there is contamination.

2.5Parameters and data analysis
This study used a non-factorial Completely Randomized Design (CRD) with 3 treatments and was repeated 10 times. Data were analyzed by Analysis of Variance (ANOVA) and DMRT test ($p<0.05$). Observations were made every day until the 30th day after inoculation (DAI). Parameters observed included: fungal contamination, bacterial contamination, browning, and callus formation.

3. Results and discussion

3.1 Fungal contamination
The data presented in this parameter include the time of the appearance of the fungus and the percentage of fungal contamination. Observations of this fungal contamination were carried out every day until the age of 30 days after inoculation (DAI). Based on ANOVA data analysis, these fungal contamination parameters showed results that were not significantly different, as shown in **Table 2.**

Table 2. Data on fungal contamination of explants

Treatment	Fungi emergence (DAI)	Percentage of fungal contamination
Sterilization Method 1	5	7.50%
Sterilization Method 2	10	5.00%
Sterilization Method 3	5	7.50%

From these data, the three sterilant methods tested showed the appearance of the fungus starting on day 5 and day 10 after inoculation. This duration is also consistent with the results of studies on the incubation period of fungi which showed that 94% of fungi appeared on day 7 and 98% on day 14 [9]. In addition, the percentage value of fungal contamination is still relatively small or safe, namely at 5%-7.5%, or from a total of 40 bottles in each treatment, only 2 or 3 bottles were contaminated with fungi.

The fungal contamination that occurred was characterized by the presence of hyphae covering the surface of the explants and media [10] (**Figure 1.**). The presence of contamination is a common problem that poses a challenge to the success of in vitro culture. Based on observational data on fungal contamination in this study the results were not significantly different, it can be said that the sterilant materials used in all methods of sterilization treatment were optimal and proven to be able to suppress the growth of fungi. Especially Fungicide at a concentration of 2g/L for 20 minutes. The use of 70% alcohol and 0.1% HgCL2 for 2 minutes was also able to suppress the growth and development of fungi. This can be seen in sterilization method 2, the fungal contamination showed 5% with a slower

emergence at 10 DAI. One study stated that HgCL2 (Mercury chloride) is highly toxic to microbes, and can cause certain concentrations [11].

Figure 1. Explants contaminated with fungi

Figure 2. Explants contaminated with bacteria

3.2 Bacterial contamination

Observations of contamination were also seen from the data of explants contaminated with bacteria. That was characterized by the presence of white mucus around the explants as shown in **Figure 2**. The data on the percentage of bacteria showed results that were not significantly different according to the ANOVA test, as shown in **Table 3.**

Table 3. Data on bacterial contamination of explants

Treatment	Bacteria emergence (DAI)	Percentage of Bacteria contamination
Sterilization Method 1	0	0.00%
Sterilization Method 2	10	17.50%
Sterilization Method 3	5	7.50%

In **Table 3**, the emergence of bacteria started from day 5 to day 10 after inoculation. However, in sterilization method 1, the explants did not experience bacterial contamination. While in the second sterilization method the percentage of bacteria reached 17.5% and the third method of bacterial contamination showed a percentage of 7.5%. In sterilization method 2, only use liquid detergent 2 ml/L, 70% alcohol, 0.1% HgCL2. It was proven that the combination did not contain any additional bactericidal ingredients so that the percentage of bacterial contamination was the highest. The possibility of this material is only able to kill bacteria on the surface, not systemically kill endogenous bacteria contained in explant tissue.

The results of bacterial contamination data showed method 1 did not grow bacteria. In the first sterilization method using Liquid detergent 2 ml/L, Dithane Fungicide 2 g/L, Agrimycine bactericide 2 g/L, Erythromycin 4 g/L, alcohol 70%, NaOCl (5.25%) 10%, and 20%. The combination of these sterilants was able to eliminate bacteria on the surface of the explants. This can be seen from the absence of bacterial contamination that occurs. Bacterial growth on culture media and explants can occur rapidly if the sterilization method is not optimal, both in the use of materials and concentrations or due to an unhygienic explant inoculation process [12], [13].

3.3 Browning

Based on observations on browning explants until 30 days after inoculation, the results of data analysis showed very significant results, as shown in **Table 4** below.

Table 4. Data on the percentage of browning explants

Treatment	Percentage of Browning	
Sterilization Method 1	0.00%	a
Sterilization Method 2	20.00%	b
Sterilization Method 3	62.50%	c

[a] The number followed by the same letter is not significantly different at ($p<0.05$) level of Duncan's test.

In method 1, it is assumed that the concentration is optimal and safe for the cells in the leaves so that it does not cause death in the leaf cells **(Figure 3)**. The use of Sodium hypochlorite (NaOCl) is a sterile material that has low activity so that the explant cells do not become stressed and injured. However, if these materials are used at high concentrations, they can cause cell injury (necrosis) and eventually die and then visually turn brown or browning [14].

Figure 3. Healthy **e**xplants from sterilization method 1

Figure 4. Partial browning explants in the sterilization method 2

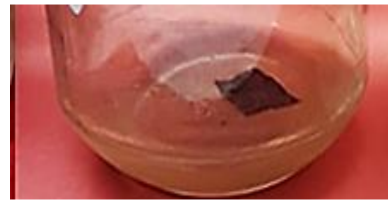

Figure 5. Overall browning explants in the sterilization method 3

In general, browning in plants occurs due to stress conditions and the oxidation process of phenolic compounds that come out due to explant injury [15], [16]. In addition, the type and concentration of sterilant ingredients are also able to stimulate the appearance of browning. As in the treatment tested, sterilization method 3 stimulated the highest explant browning, namely 62.5% **(Figure 5)**, and sterilization method 2 stimulated 20% browning **(Figure 4)**. The use of HgCL2 or Mercury Chloride was suspected to be the cause of browning in treatments 2 and 3 because both treatments used HgCL2 in the series of sterile materials. Mercury Chloride is a sterilant that is highly toxic not only to microbes but also to explants and humans. Explants that were sterilized in treatments 2 and 3 were seen to have browned in some of the explants. However, some had all of the explants browned. Browning is one sign that the explant cells are dead [17].

Mercury Chloride is highly corrosive and at high concentrations can inhibit enzyme activity and cause protein precipitation in microbial cells and plant cells, causing damage to plant tissues. The use of $HgCl_2$ at high concentrations has also been shown to cause browning and death of sugarcane leaf explants [18].

3.4 Callus formation

Observations on callus formation were carried out every day until the 30th day after inoculation. The results of ANOVA data analysis showed that the percentage of explants that succeeded in forming callus and the score of callus formation on the circumference of the explants showed very significant results, as shown in the data listed in **Table 5.**

Table 5. Callus formation data

Treatment	Callus emergence (DAI)	Percentage of callus formation		Callus formation score on the circumference of the explant	
Sterilization Method 1	11.67	85.00%	a	3 (>50%-75%)	a
Sterilization Method 2	15.30	45.00%	b	1 (0-25%)	b
Sterilization Method 3	16.00	35.00%	b	1 (0-25%)	b

[a] The number followed by the same letter is not significantly different at ($p<0.05$) level of Duncan's test.

Observational data showed that the tested sterile materials also affected callus formation and growth. This can be seen in the treatment of sterilization method 1 which showed the appearance of callus for the first time at 11.67 DAI, and an average of 85% of the explant cells was able to regenerate to form a callus and the growth was relatively even, with a score of 3 or about 75% around the explants able to form a callus.

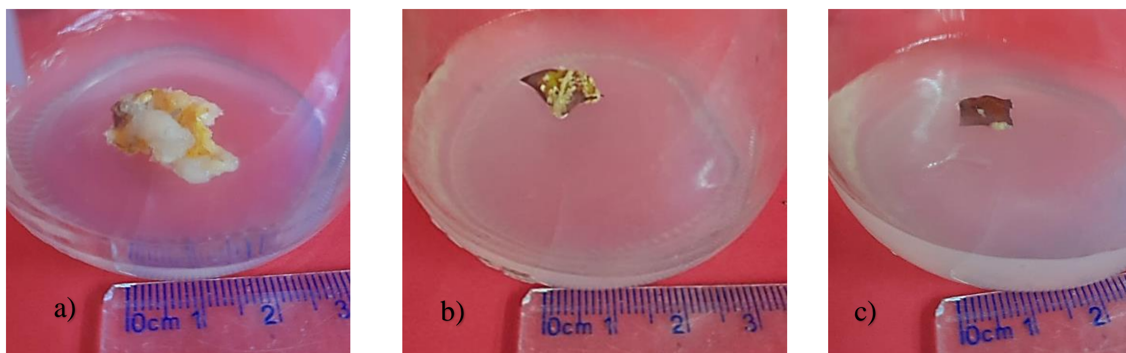

Figure 6. a) Callus growth score 3 on sterilization method 1; b) Callus growth score 1 on the 2nd sterilization method; c) Callus growth score 1 in the 3rd sterilization method

Different results were shown in the treatment of sterilization methods 2 and 3 which were relatively slower to produce callus, namely at 15-16 DAI with a percentage between 35%-45% of explants capable of forming a callus. However, the callus formed was uneven, only in small spots on the explants that did not experience browning.

The difference in the response of callus formation to the treatment of sterile materials was thought to be due to stress and cell necrosis caused by each treatment, especially from methods 2 and 3. The recovery process of explant cells affected cell formation. This can be seen in the treatment of method 1 which seems to be able to form callus faster.

In addition, browning on some of the explant surfaces also affected callus formation. Based on the results of observations callus appeared on the explants that did not experience browning. It is known from the data of this study that the treatment of sterilization methods 2 and 3 on average experienced browning of explants so that only certain parts of the explants grew callus. Based on the score, it only reached 25% of the total circumference of the explant piece. The browning effect on explants resulted in inhibited absorption of growth regulators and nutrients [19], so that the cells were unable to regenerate to form callus.

4. Conclusions

The conclusion of this study shows that sterilization method 1 is the optimal method to suppress fungal and bacterial contamination. On average, fungi and bacteria appeared on day 5 after inoculation. In addition, the treatment of several sterilization methods greatly affected the browning of the explants. In sterilization method 1 there was no browning of explants, while other methods caused browning to reach 62.5%. Browning also causes differences in callus regeneration ability. Explants sterilized by method 1 showed a high percentage of callus (85%) and callus appeared more quickly at 11 days after inoculation.

5. Acknowledgements

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