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IN VITRO STERILIZATION METHOD OF THE BANYUWANGI'S LOCAL RED DURIAN LEAF EX PLANTS TO SEVERAL COMBINATION TYPES OF STERILIZATION MATERIALS

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ABSTRACT

This research aimed to determine the most appropriate explant sterilization material in in vitro propagation of red durian. By doing so, it may be able to determine the successful research and become the research focus in obtaining the standard method of sterilization. Furthermore, this research was held in January to May 2018 at the Tissue Culture Laboratory of Universitas Jember. The planting material used was red durian Sunrise of Java (SOJ) plant originated from Songgon, Banyuwangi, East Java, Indonesia. Specifically, the planting material used was young foliage at the axillary bud, which was the result of one year mini grafting. This research used 7 sterilization methods (P1 – P7), whereas each of them was processed by combining several sterilization materials such as Clorox, beta dine, detergent, alcohol 70%, bactericide, fungicide, tween 20, and dettol. Moreover, the sterilized explant was planted in MS 0 media and incubated in a 20-25 ℃ room with the light intensity of 1000 – 2000 lux. After that, the researches observed it for 10 – 14 days. The parameter of such observation includes: living explants percentage (%), contaminated explants percentage (%), and browning explants (%). The result of this research reveals that, among the others, P7 is the best treatment since contaminated explants were not found. However, such treatment did not have any living explants because 100% of them were browning.

Keywords: Red durian **,** Sterilization, Clorox, Detergent

1. INTRODUCTION

Red durian is one of the excellent commodities in Banyuwangi Regency since it has its own exotic value, and is categorized as endemic. This plant is presumably the derivative of natural hybrid between *D. zibethinus* and *D. graveolens*. Banyuwangi's local red durian has several distinctive characters i.e. red flesh, typical flavor with soft aroma, thicker flesh, small-sized seed, low alcohol content, high anthocyanin content, high phytosterol and phytohormone proteins functioned as anti stress, anti hypertension, and aphrodisiac (Rusmiati, dkk., 2013).

Despite of the fact that red durian is Banyuwangi's Regency featured commodity and highly potential to be developed commercially, the unavailability of qualified seedling in a sufficient

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amount turns out to be the main obstacle. Sudjijo (2011) states that qualified seedling is one of the main production components in a plant cultivation.

However, conventionally cultivated red durian still face some obstacles. Generally, the commonly used technique of Banyuwangi's local red durian propagation is by using the seed while, in case of the vegetative propagation, is by using mini grafting technique. According to Sukarmin (2010), the seed propagation typically results in higher success rate than the other vegetative ones. Also, seed propagation does not require any specific expertise and only needs nursery skills. But, for the seedling originated from seed, the result is more likely to be unsatisfied due to the high level of heterozygosity. Such technique is not also recommended since its fruit characteristics is frequently deviated from its parent. Therefore, seed propagation will result in diverse plant due cross pollination.

Other than by seed, red durian may be vegetatively propagated by mini grafting technique. However, this propagation technique may not provide a large amount of red durian seedling yet, since the amount of buds and readily grafted branches are limited.

According to Hambali *et al* (2009), plant propagation by tissue culture (in vitro) offers the big chance of producing a large amount of seedling in a relatively short time that result in economical benefit. This plant propagation technique may be adopted throughout the year and does not depend on the seasons. Zulkarnain (2009) also contends that tissue culture has its own benefit, i.e. massive propagation of even and good quality red durian seedling, in which its character is as good as its excellent parent. By using this technique, million clones may be produced only from a small number of initial material within a year.

Tissue culture refers to a method of isolating particular part of plant such as cell, tissue, and organ and grow them as a complete plant in aseptic environment (in vitro). The successful practice may highly be affected by the used media such as explant source, application of growth control substance, macro and micro nutrient, organic material, carbohydrate, amino acid, vitamin, media's compaction material and material condition, and sterilized room. The growth response of planlet at the tissue culture also depends on the type of the cultured plant (Narayanaswamy, 1994).

According to Gunawan (1992), it is greatly necessary to prevent and avoid the explants from being contaminated in all of the activities of tissue culture technique. This aspect seriously determines the technique's success in the plant propagation by tissue culture technique. In order to prevent and avoid the explants form being contaminated, sterilization was applied. The activity of explants sterilization aimed to eliminate the microorganism that may be brought when collecting the explants, in which it may result in contamination that can inhibit the explants from being a complete plant. There are many disinfectants that may be used to sterilize the media in tissue culture, one of the most commonly ones used is NaClO.

This research aimed to determine the most appropriate sterilization material for explants in the in vitro propagation of red durian. Figuring out such material will allow the researchers to determine the success of red durian by in vitro propagation and use it as the research focus to obtain the standard method of sterilization.

2. RESEARCH METHOD

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2.1 Time and Place

This research was held in January to May 2018 at the Tissue Culture Laboratory of Universitas Jember.

2.2 Plant Material

Red durian is originated from Songgon, Banyuwangi, East Java, Indonesia. The plant material used in this research was the seedling of Banyuwangi's local red durian SOJ that was the result of one year mini grafting (Figure 1a). Also, the plant material used as the explant source was the young leaves adjacent to the axillary bud (Figure 1b).

Figure 1. (a) Seedling of red durian SOJ; (b) Source of red durian explants

2.3 Explants Sterilization

The explants of Banyuwangi's local red durian leaves were sterilized by applying 7 sterilization methods presented in Table 1.

Table 1. Sterilization Method Combination of Red Durian Explants

No.	Sterilization Method	Code	Time (Min)
1	Running water	P1	30
	10% Clorox		3
	5% Clorox		3
	70% Alcohol		3
2	Running water	P2	30
	10% Clorox		3
5	Detergent	P5	30
	2gr/250ml Bactericide+fungicide		60
	Running water		30
	70% Alcohol		1
	20% Dettol		10
	10% Dettol + 3 drops of Tween 20		5

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	5% Clorox		3
	70% Alcohol		3
	2,5% Betadine		5
3	Detergent	P3	10
	20% Clorox		5
	10% Clorox		5
	70% Alcohol		2
	Bactericide+fungicide		30
4	Running water	P4	30
	2gr/250ml Bactericide+fungicide		60
	5% Clorox		15
	20% Clorox		10
	70% Alcohol		1

	5% Betadine		10
6	Detergent	P6	10
	70% Alcohol		1
	2gr/250ml Bactericide+fungicide		60
	5% Betadine + 2 drops tween 20		10
7	Detergent	P7	10
	Running water		30
	70% Alcohol		10
	50mg/L Antibiotic		60
	20% Clorox		5
	10% Clorox		5
	70% Alcohol		1
	5% Betadine		5

First procedure :

First procedure (P1) : the explants were washed by running water for 30 minutes. The clean explants were sent to the Laminar Air Flow Cabinet. Such explants then were soaked in 10% and 5% NaClO (Clorox) for 3 minutes. Next, they were soaked in 70% alcohol for another 3 minutes.

Second procedure (P2) : the treatment in this procedure is nearly similar to the treatment in P1. The explants were soaked in 70% alcohol for 2 minutes then in 2.5% betadine for 5 minutes.

Third procedure (P3) : the explants were washed with soft detergent and rinsed with running water for 10 minutes. The clean explants then were brought to the Laminar Air Flow Cabinet. The procedure performed in Laminar Air Flow Cabinet was soaking the explants in 20% and 10% clorox solution, 5 minutes for each, then in 70% alcohol for 5 minutes, continued with 2% bactericide and fungicide solutions for 30 minutes.

Fourth procedure (P4) : the explants were washed with running water for 30 minutes. Next, it was soaked in bactericide and fungicide solutions (2gr/250ml) for 1 minute. Subsequently, it was drained and brought to the Laminar Air Flow Cabinet. In that LAF, the explants were soaked in 5% and 2% Clorox solution for 15 minutes and 10 minutes respectively. After that, they were soaked in 70% alcohol for 1 minute.

Fifth procedure (P5) : the explants were washed with soft detergent and rinsed with running water for 30 minutes. Afterwards, they were dipped in 2 gr/250 ml fungicide and bactericide solutions for 1 hour then rinsed in running water for 30 minutes. The clean explants were

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brought to the Laminar Air Flow Cabinet. The procedure performed in the Laminar Air Flow Cabinet was soaking the explants in 70% alcohol for 1 minute and in 20% Dettol solution for 10 minutes. Subsequently, they were soaked in 10% Dettol solution added with 3 drops of tween 20 for 5 minutes before soaking them in 5% Betadine for 10 minutes.

Sixth procedure (P6) : the explants were washed with running water for 10 minutes and soaked in 70% alcohol for 1 minute. Next, they were soaked in 2gr/250ml bactericide and fungicide for 1 hour. After that, the explants were drained and brought to the LAF. In the LAF, they were soaked in 5% betadine added with 2 drops of tween 20 for 10 minutes.

Seventh procedure (P7) : the explants were soaked in soft detergent for 10 minutes and rinsed with running water for 30 minutes. Subsequently, they were soaked in antibiotic for 1 hour and brought to LAF. In the LAF, they were soaked in 20% and 10% Clorox solution for 5 minutes for each. Afterwards, they were soaked in 70% alcohol for 1 minute and 5% betadine for 5 minutes.

After completing the sterilization stage in the combination of P1 – P7 method, each explant from each method was rinsed 3 times with sterilized aquades for 10 minutes for each rinsing. Afterwards, the planting material was ready to be planted in in vitro media.

2.4 Explant Planting

The sterilized explants were planted in MS 0 media and incubated in a 20-25 °C room with 1000 – 2000 lux light intensity and observed for 10 – 14 days. The parameter for the method of explants sterilization includes :

a. Living explants percentage (%)
   The living explants may be recognized from their freshly green leaves and response to the swelling explant leaves. The living explants percentage (%) was calculated by using the following formula :

$$\%\ living\ explants = \frac{number\ of\ living\ explants}{total\ explants}\ x\ 100\%$$

b. Contaminated explants percentage (%)
   Contaminated explants may be caused by fungi or bacteria. Fungal contamination is characterized by the white soft lines while the bacterial one is by slimy patches on the media or explants. The formula used to determine the contaminated explant percentage (%) is :

$$\%\ contaminated\ explants = \frac{number\ of\ contaminated\ explants}{total\ explants}\ x\ 100\%$$

c. Browning explants percentage (%)
   Browning refers to a state when the explants turn brown or black, which result in the growth or development or the dead explants. The browning explants percentage (%) is :

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$$\%\ browning\ explants = \frac{number\ of\ browning\ explants}{total\ explants}\ x\ 100\%$$

3. RESULT AND DISCUSSION

There were 20 explants used to each treatment. The observation result in the sterilization method showed different responses to each treatment. As stated by Gunawan (2007), the initial stage in the successful tissue culture activity was the explants sterilization. If such activity was unsuccessful, it may not give any benefit. The obstacles occurred during the sterilization if the explants were originated from the field, limited, and no supporting information from the previous studies (new plant). The explants originated from the field mainly contained dirt or microorganism that made the plant is both externally (surface) and internally (within the tissue) prone to contamination. For the new plant, specifically when the explants used are limited, the effective and efficient exploration or special treatment are deemed necessary. There are two types of explants sterilization, mechanical or chemical. The sterilization applied in this research were chemical. The chemical material utilized were fungicide, bactericide, bayclin, HgCl2, antibiotic, and alcohol. The result of sterilization to the leaves explants with there parameters for the observation of explants sterilization are presented in Table 2.

Table 2 Response of red durian explants to seven combinations of in vitro sterilization methods

Code	Sterilization Method	Time (Min)	Explants Response (%)			
			Explants number	Living	Browning	Contaminated
P1	Running water	30	20	0	0	100
	10% Clorox	3				
	5% Clorox	3				
	70% Alcohol	3				
P2	Running water	30	20	0	5	95
	10% Clorox	3				
	5% Clorox	3				
	70% Alcohol	3				
	2,5% Betadine	5				
P3	Detergent	10	20	0	10	90
	20% Clorox	5				
	10% Clorox	5				
	70% Alcohol	2				
	2% Bactericide+fungicide	30				

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P4	Running water	30	20	0	5	95
	2gr/250ml Bactericide+fungicide	60				
	5% Clorox	15				
	20% Clorox	10				
	70% Alcohol	1				
P5	Detergent	30	20	0	10	90
	2gr/250ml Bactericide+fungicide	60				
	Running water	30				
	70% Alcohol	1				
	20% Dettol	10				
	10% Dettol + Tween 20, 3 drops	5				
	2,5% Betadine	10				
P6	Detergent	10	20	0	15	85
	70% Alcohol	10				
	2gr/250ml Bactericide+fungicide	60				
	tween 20, 2 drops + Betadine 2,5%	10				
P7	Detergent	10	20	0	100	0
	Running water	30				
	70% Alcohol	10				
	50mg/L Antibiotic	60				
	20% Clorox	5				
	10% Clorox	5				
	70% Alcohol	1				
	5% Betadine	5				

Based on Table 1, in the first treatment (P1), the percentage of the biggest contaminated explants are 100%. This may occur since the combination between 10% and 5% Clorox and 70% alcohol are not suitable for the explants sterilization of red durian leaves. This shows that the use of explants sterilization material highly determine the in vitro plant technique. The use of the sterilization material combination is also not suitable so that it resulted in contaminated explants and unsuccessful in vitro propagation for durian plant. NaClO (Clorox) are commonly used as the whitening material or disinfectant. This compound can eliminate bacteria and virus. In the

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tissue culture technique, such compound is generally used as the sterilization material for the plant's tissue surface (Sawant and Tawar, 2011). NaClO is also able to clean up the microorganism brought in the plant material, eliminate the particles of soil, dust, and others (Santoso dan Nursandi, 2003). The use of NaClo as the sterilization material for the surface of numerous plant's explant sources has been frequently reported (Morla et al., 2011). Since it only acts as as the sterilization material for the plant's tissue surface, the effectiveness of NaClO in controlling the explants contamination is also low. If this compound is given in low concentration and exposure time, the result will not be too effective, especially in controlling the explants contamination (Farooq et al., 2002). In other words, the less the NaClO concentration, the more the explants are prone to. However, higher NaClO concentration may inhibit the development of explants tissue (Rismayani dan Hamzah, 2010).

In P2, the treatment performed was nearly similar to the one in P1 and the researchers only added 2.5% Betadine for 5 minutes at the end of the treatment. The differences between P1 and P2 are presented in Table 1, whereas the P2 shows the decrease of the contaminated explants amounted to 90% while the rest were the browning ones amounted to 10%. This is caused by the Betadine's active material, in this case is Povidon-iodin, which is the antimicrobial substance with the broadest spectrum that is able to eliminate bacteria, fungi, protozoa, and virus (Williams dan Wilkins, 2006).

In P3 and P5, the contaminated explants are amounted to 90%, while the browning ones are 10%. In P4, the contaminated explants are amounted to 95% while the browning ones are 10%. Meanwhile, the sixth treatment (P6) shows better result than P1, P2, P3, P4 and P5 because, as presented in Table 1, the percentage of the contaminated explants are amounted to 85% while the browning ones are 15%. According to Wahyudi (2014), the explants turn brown because of the impact of the swelling and this condition will inhibit the tissue development. Adri (2012) contends that the dead browning explants are caused by phenol oxidation.

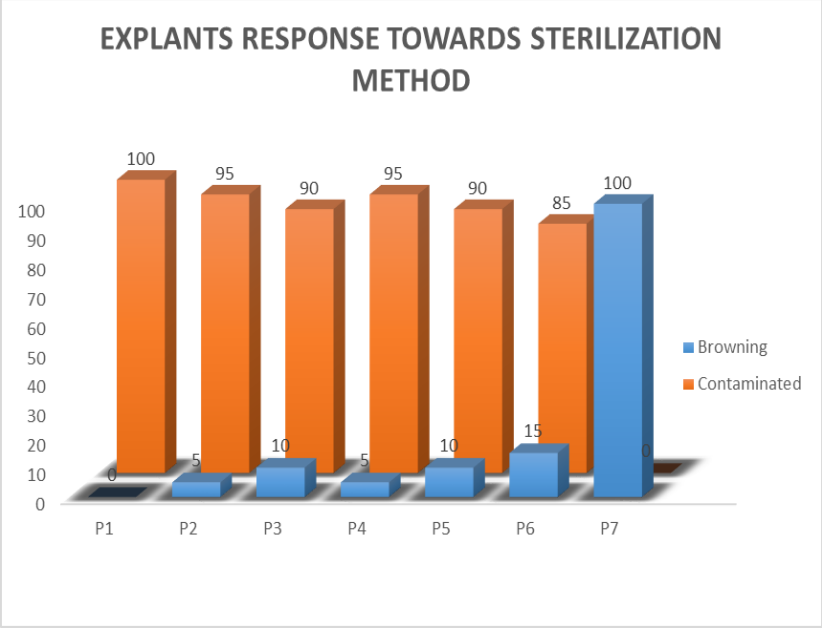

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Figure 2. Explant Response towards Sterilization Method

In this research, eventhough it did not have living explants, the seventh is considered as the best treatment since there were no contaminated explants. Figure 2 shows that contaminated explants in P7 is amounted to 0% while the browning ones are 100%. There is no specific standard on the duration and percentage of the sterilant application, since this depends on each explant. As stated by Gunawan (1992), the common duration of sterilant application to the explants should not be too long because its toxic material may affect the plant tissue, including explants. Therefore, based on the result of this research, it is possible to poison and eliminate the explants that had been soaked for 5 minutes in the sterilant material eventhough they were free from the fungi and bacteria.

Generally, the sterilant is toxic and able to eliminate the plant tissue. Rismayani and Hamzah (2010) state that the small concentration of sterilant will inhibit the development of planlet tissue in Aglaonema sp. Also, the hypochlorite solution (natrium and calcium) has proved that it is able to solve the contamination to the surface of several plants. As reported by Rismayani and Hamzah (2010), the utilization of sterilant, in this case is 3% clorox, may perfectly sterilize the tissue in Aglaonema sp. and increase the number of buds.

Other than the sterilization material, the other factor causing the failure of sterilization method is the explants themselves since each of them may have different surface contamination levels. Zulkarnain (2009) affirms that the explants size may affect their living response so it will impact the successful tissue culture. The small size of explants will affect their physiological function. This may cause explants' quick death. The small sized explants have insufficient content of metabolite compound to balance the growth control substance given to the media so that they can only adapt and provide swelling response before they die.

Other than the size, the explants morphological surface, hairy or bare, may also affect the sterilization process. Based on the leaf explants observation of this research, the researchers found that the red durian SOJ leaves have a large amount of soft hair (Figure 3 and 4). Those hair is in compliance with Tjitrosoepomo's statement in 2005 that the upper surface of the leaf is glowing (Figure 3), while the lower one is hairy and brownish (Figure 4). According to Zulkarnaen (2009), the use of 70% alcohol and addition of detergent and 1-2 drops of 20 tween aims to allow the surface of disinfectant material touches the small bends or cavity that look like gap among the explant's soft hair so that the explant is be completely sterile. This may result in more effective sterilization.

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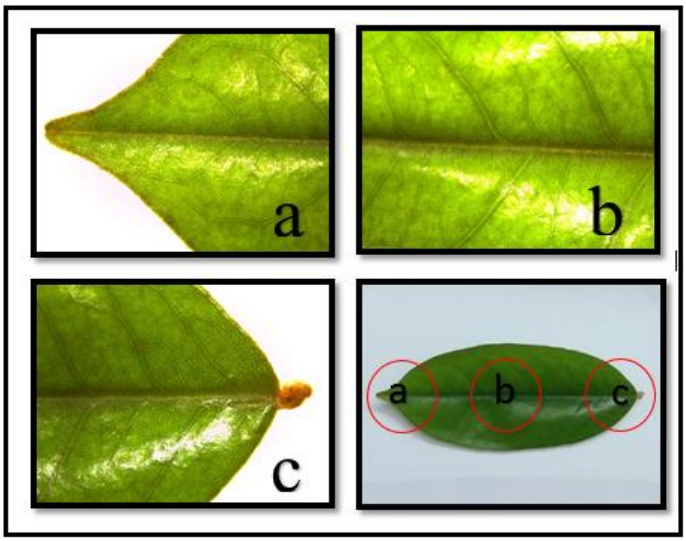

Figure 3. Upper part of leaf under microscope "LEICA EZ4 HD" with 8X magnification, (a) tip; (b) midrip; (c) petiole

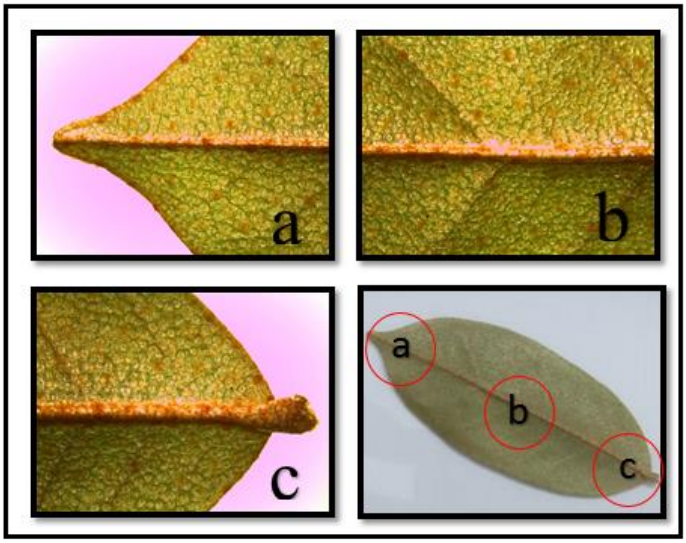

Figure 4. Lower part of leaf under microscope "LEICA EZ4 HD" with 8X magnification, (a) tip; (b) midrip; (c) petiole

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CONCLUSION

The result of this research shows that P7 is the best treatment since it has no contaminated explants. However, it also has no living explants because 100% of them are browning.

SUGGESTION

The researchers suggest further research to figure out the appropriate method of explants sterilization treatment in the in vitro propagation of red durian

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