The optimization of in vitro sterilization on Ebony (*Diospyros celebica* Bakh) using shoot explants

A Dalauleng¹, Gusmiaty², F D Panannangan³ and M Restu²

¹The students of Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Universitas Hasanuddin, Makassar
²Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University, Makassar
³2nd Regional of Seed/Seedling Forest Tree, South Sulawesi, Indonesia

Email: umyhody@gmail.com

**Abstract.** Ebony (*Diospyros celebica* Bakh) is a woody plant that has been used in the manufacture of luxury veneers, plywood, luxury furniture, sculpture, carving decorative tools, sticks, piano guitars, wind instruments and building materials. To maintain the existence of Ebony, it is necessary to provide good seedling quality through the tissue culture method. Tissue culture is one of the clonal propagation techniques for mass propagation. Moreover, it is able to produce a large quantity of plants in a relatively short time and obtain superior seedling. The process of sterilizing plant material (explants) is an important activity in tissue culture which aims to eliminate microorganisms carried during explant extract, which can cause contamination. This study aimed to obtain the optimal sterilization procedure for Ebony in vitro micropropagation. The analysis used was descriptive analysis by observing the percentage of contaminated explants, types of contamination, and explants that had browned (browning). Sterilization using Bactericides and Fungicides showed the lowest contamination level (71%). There were three types of contamination found, namely fungal contamination, bacterial contamination, and a combination of fungal and bacterial contamination. The lowest percentage of browning explants was using sterilization 3 (71%).

1. Introduction

Ebony (*Diospyros celebica* Bakh.) is a type of timber or wood that has a local name of Ebony and belongs to the Genus Ebenaceae [1]. This species is a forest timber plant with high economic value [2]. Ebony in the industrial forest program is one of the tree species that is chosen in the development of government programs [1].

The amount of supply does not balance the increasing demand for Ebony. As a result, it increases the price of Ebony in foreign markets from year to year. Furthermore, the logging of Ebony without ecological considerations affects the plants that will be exploited because they are not ready to be harvested yet. Thus, it is very detrimental to the sustainability of production.

IUCN (2003) has noted the Ebony into the vulnerable category by the IUCN Red List Categories of Threatened Species, which is defined as a high risk of extinction in nature. This species has been evaluated for inclusion in Appendix II CITES as the endangered species if there are no strict rules governing its trade [3]. Some research of ebony focused in genetic diversity and mating system [4,5].

The strategy used to minimize the threat of extinction in Ebony is by using tissue culture methods that can cultivate superior Ebony. Tissue culture is a technique of cultivating cells, tissues, or organs
of a plant under aseptic conditions (free of all forms of microorganisms) and in a controlled environment [6].

Some benefits obtained from tissue culture are to get new plants in large quantities in a relatively short time, to have the same physiological and morphological characteristics as its parent, and it is also expected to obtain the new and superior quality of plant [7]. According to Zulkarnain (2011) [8], the main benefit of plant tissue culture technique is to multiplicate the clones or mass propagation of plants that are genetically identical to each other.

Tissue culture techniques will succeed if they meet the requirements of tissue culture. The process of explant sterilization plant material is one of the crucial activities in tissue culture. The purpose of explant sterilization is to eliminate microorganisms carried during the extraction of explants that can cause contaminations that can inhibit explant growth [9]. The stages carried out in the explant sterilization process are the sterilization of the work environment, sterilization of media, and preparation of explant sterilization [10].

These are some previous studies on the effect of sterilization techniques and medium composition on the growth of Sirsak ratu shoots. It was carried out with two stages of sterilization that showed the explants with the lowest levels of contaminants and browning; then, it could induce the best shoots. Cahyani’s research, (2017) [11] showed that the optimal sterilization explant used in teak was sterilization using bactericide (Agrept 20 WP) and fungicide (Masalgin 50 WP).

This research needed to conduct because it could optimize the sterilization process of Ebony shoots as the explants. The results of this study could be useful in overcoming the problem of ebony explant contamination so that the optimal growth can be obtained.

2. Research methodology

2.1. Research material

The plant material used was ebony shoots obtained from the two-year-old ebony seedling. Ebony seedlings were taken from 2nd regional of Seed/Seedling Forest Office. The medium used in this study was Murashige and Skoog (MS) media.

![Shoot](image1)

**Figure 1.** Two-year-old seedling of Ebony

2.2. Explant sterilization procedure

The preparation of explants was carried out through the following stages:
Figure 2. The shoot explant

a. The explant was taken from a two-years-old ebony seed.
b. The Ebony shoots were cut to a size of ± 4 cm.
c. The cutting shoot was stored in containers containing sterile distilled water and two drops of tween 20 to reduce phenolic.
d. The remnants of the leaves at the shoots were cleaned and washed using detergent while being brushed under running water.
e. Then, the explants were rinsed using sterile aquades three times.
f. The sterilization was continued based on each treatment.

2.3. Research Design
This research was organized into three explant sterilization stages which consisted of:

2.3.1. Sterilization I.
a). The explants were immersed in a 2% Masalgin Fungicide solution for an hour then rinsed with sterile distilled water three times.
b). Then, the explants were immersed in a 2% Agrept bactericidal solution for an hour then rinsed with sterile aquades three times.
c). In LAFC, the explants were soaked in 70% alcohol added two drops of tween 20 solutions for 10 minutes.
d). The explants were rinsed three times using sterile distilled water.
e). The explants were soaked again in 50% Clorox added two drops of tween 20 solutions for 10 minutes.
f). The explants were rinsed using sterile aquades three times.
g). Then, the explant was immersed in 0.1 ml Plant Preservative Mixture (PPM) added two drops of tween 20 solutions for 5 minutes.
h). The explants were rinsed using sterile aquades three times.
i). Then, the samples were cut to ± 2 cm in size and dried in a petri dish on which sterile filter paper had been placed.
j). After drying, the explants were ready to be planted.
2.3.2. Sterilization II.

a). The explants were immersed in 2% Dithane M-45 solution for 1 hour and then rinsed with sterile aquades three times.

b). The explants were immersed in two drops of Dettol and 1 ml of detergent solution for 1 hour then rinsed three times using sterile distilled water.

c). In LAFC, the explants were immersed in 70% chloride added tween 20 solutions for 15 minutes; then, the explants were rinsed three times using sterile distilled water.

d). The explants were immersed again in 30% Clorox added two drops of tween 20 solutions for 5 minutes, then

e). The explants were rinsed using sterile aquades three times.

f). Next, the explants were cut to ± 2 cm in size and dried in a petri dish on which had been placed a sterile filter paper.

2.3.3. Sterilization III.

a). The explants were immersed in 2% Dithane M45 solution for 1 hour, then rinsed with sterile distilled water three times.

b). The explants were immersed in Agrept 2% bactericidal solution for 1 hour, then rinsed with sterile aquades three times.

c). The explants were immersed in 70% alcohol solution for 3 minutes; then, the explants were rinsed three times using sterile distilled water.

d). In LAFC, the explants were immersed again in 50% Clorox added two drops of tween 20 solutions for 5 minutes.

e). The explants were rinsed using sterile aquades three times.

f). The explant was cut to size ± 2 cm in a solution of Vitamin C ampoules.

g). Then, the explants were rinsed using sterile distilled water two times.

h). The explants were dried on a petri dish on which sterile filter paper had been placed.

i). After drying, the explants were ready to be planted.

2.4. Data analysis

The analysis used in this study was a descriptive analysis with bar charts.

2.4.1. The Observation Variable

a) The percentage of contaminated explants. The observation was done every day and calculated with the formula:

\[
\frac{\sum \text{Number of contaminated explant}}{\sum \text{Total number of planted explant}} \times 100\%
\]

b) The percentage of contamination type. The type of contamination consisted of two bacterial contamination and fungal contamination. The percentage was calculated using the following formula:

\[
\frac{\sum \text{Number of each contamination type}}{\sum \text{Total number of planted explant}} \times 100\%
\]

c) The percentage of browning explants. The percentage was calculated every day by the following formula:

\[
\frac{\sum \text{Number of browning explant}}{\sum \text{Total number of planted explant}} \times 100\%
\]
3. Results and discussion

3.1. Percentage of contaminated explants

Contamination is the main problem that often arises in tissue culture. In general, there are two contaminations: bacterial contamination and fungal contamination. Based on data that had been obtained from 3 sterilization treatments, it could be seen that from 21 explants planted, 19 of them experienced the contamination. The average percentage of contamination per treatment are presented in Figure 3.

![Figure 3. The Percentage of Contamination for Each Sterilization Treatment in Ebony Tissue Culture.](image)

The diagram (Figure 3) shows that the sterilization 1 (bactericidal and fungicidal sterilization) obtained the lowest level of contamination (71%). This was due to the use of bactericides and fungicides that inhibit the growth of fungi and bacteria on the surface of the explant. However, there were some explants which experienced the contamination. According to Sembiring in Nasution (2013) [12], the use of fungicides and bactericides can be the inhibitors that inhibit the growth of fungi and bacteria against explants.

The sterilization 2 (Masalgin and Dettol + detergent) showed 100% of contamination rate. The sterilization using antibiotics initially experienced a good growth response, but a few days later, the explants were contaminated. According to Yuliarti (2010) [13], the use of antibiotics in plant tissue culture was less successful. There were no effective antibiotics to kill the microorganisms that cause contamination.

The sterilization 3 using Dithane M-45 fungicide and bactericide showed a 100% contamination level because the Dithane M-45 fungicide was only able to kill the fungus but was unable to kill the bacteria present in the explant. Zulkarnain (2011) [8] stated choosing a method of sterilization must be selective in order to eliminate bacteria or fungi of the explants. To be compared to other sterilizers, sterilization one was the best of all, with the lowest level of contamination of 71%. Cahyani's study (2017) [11] reported the use of Bactericides (Agrept 20WP) and Fungicides (Masalgin 50WP) could inhibit the growth of fungi and bacteria on the surface of explants. Thus, the cultured explants were able to grow into complete plants, even though some of them were still contaminated.

The sterilization 1 had the contamination at the 4th observation (10 days after planting). The sterilization 2 began to experience contamination at the third observation (8 days after planting). In sterilization 3, the contamination began to occur at the 4th observation (10 days after planting).

An alternative approach for the improvement of this crop is to complement traditional breeding methods like *Gignathocloa atter* [14] with biotechnology techniques [15] to regenerate plants from single cells and organized tissues and to transfer desirable genes from other sources.
3.2. The percentage of contamination type

Contamination in this study was generally caused by fungi and bacteria. The symptoms caused by fungi were hypha on the surface of the media and explants. In addition, the hypha was white until it turned black. The contamination caused by bacteria was characterized by the appearance of yellowish-white mucus on the surface of the media and explants.

Figure 4. The percentage of contamination type for each sterilization treatment in Ebony tissue culture (blue: fungus, green: bacteria, red: fungus + bacteria)

The percentage of contamination type (Figure 4) shows that 42% of the fungus contamination was mostly found in the sterilization 2 (Masalgin and Dettol + detergent), while the sterilization 1 (bactericides and fungicides), and 3 (Dithane M-45 and bactericidal), as much as 14%, respectively. To be compared to other sterilizers, sterilization one was the best among others for treating fungal contamination. The type of bacterial contamination showed that this type of contamination was only found in sterilization two as much as 28%. This observation was in line with the opinion of Yuliarti (2010) [13] which stated that the use of antibiotics in plant tissue culture is less successful because there is no effective antibiotic to kill microorganisms that cause contamination, but in the sterilization 1 (Figure 4), found a type of combined contamination between bacteria and fungi. So, among the three types of sterilization treatments, no one was able to kill bacteria and fungi simultaneously. Zulkarnain (2011) [8] stated that in choosing a method of sterilization must be selective, so that in eliminating unwanted bacteria or fungi with minimal interference with explants.

3.3. The percentage of browning explants

Explant tissue cultures often turn brown (browning), which can inhibit growth and ultimately cause tissue death. Browning is ubiquitous in woody plant species. Explants often exhibit browning symptoms around explant pieces. This situation is caused by the oxidation of phenolic compounds produced by plant tissue.

The percentage of browning explants in the three sterilization treatments used variations in Chlorox concentration and immersion time with different concentrations in each treatment. Chlorox is used in sterilizing explant surfaces because it functions as a disinfectant. Yuliarti’s study (2010) showed the 50% of explants that were not treated with chlorox on the first day after planting were brown on the surface of the cutting side and secreted brown liquid. On the second day, the liquid started seeping down, causing the explants affected by the liquid to turn brown. The longer this liquid spread, the explant part could be rotten and eventually dried.
Sterilization 1 and 3 used 50% chlorox, and sterilization 2 was with concentrations of 70% and 30%. According to Hendaryono and Wijayani (1994) [7], chemicals often used for surface sterilization of explants include Sodium Hypochlorite, such as Clorox and Bayclin (commercial bleaching product). The concentration for sterilization depends on the softness of the explant.

Figure 5. The Percentage of Browning for each sterilization treatment in Ebony tissue culture.

The result of observation on the browning percentage (figure 5) shows that the sterilization one and sterilization 2 were 100%, respectively. The percentage of explants that experience browning on sterilization 3 was lower at 71%. Sterilization 3 was the best treatment compared to other sterilizers. On the treatment of sterilization 1, the explants began to experience browning at the second observation (6 days after planting). In the 2nd sterilization treatment, the explants began to experience browning at the third observation (8 days after planting). However, in sterilization 3, explants began browning at the 4th observation (10 days after planting).

4. Conclusion
The most optimal sterilization of Ebony shoot explant was sterilization, one which was using Bactericides and Fungicides with the lowest contamination rate of 71%. Then, the lowest percentage of an explant that experienced browning was 71% on sterilization 3.

References
[1] Darusman D 2001 Kajian produksi, perdagangan, industri dan teknologi eboni. Makalah Pemabahasan pada LokakaryaManajemen LokakaryaManajemen Eboni dalam Mendukung Keunggulan IndustriMenuju Otonomisasi dan Era Pasar Bebas (Makassar)
[2] Soerianegara 1967 Beberapa keterangan tentang jenis-jenis eboni Indonesia pengumuman no. 92 (Bogor: Lembaga Penelitian Hutan)
[3] Nurkin, Allo B and Gintings 2018 Eboni Sulawesi (Makassar: UPT Unhas Press,3.)
[4] Larekeng S H, Restu M, Susilowati A and Rachmat H H 2019 Genetic diversity of parental and offspring population in ebony (Diospyros celebica bach) revealed by Microsatellites marker Int. J. Emerg. Technol. 10 178–85
[5] Restu M, Gusmiaty G and Larekeng S H 2017 High outcrossing rate and pollen dispersal distance of Diospyros celebica Bakh . ( EBENACEAE ) , an endemic tree species in sulawesi island , Indonesia Biotropia (Bogor). 24 173–81
[6] De E, Jod C and A K 2003 dPlant Cell Tissue (London (UK): Bios Scientific Publisher)
[7] Hendaryono and D.P.S Wijayani 1994 Teknik Kultur Jaringan (Yogyakarta: Kanisius)
[8] Zulkarnain Z 2009 Kultur Jaringan Tanaman: Solusi perbanyakan tanaman budi daya (Bumi Aksara)
[9] R B, Larekeng S H, Arsyad M A, Gusmiaty G and Restu M 2020 In vitro growth response on
three provenances of Jabon Merah based on auxin and cytokinin combinations *IOP Conf. Ser. Earth Environ. Sci.* 486 012088 486 1–16

[10] Suratman S K, Pitoyo A and Mulyani S 2013 *Keefektifan Penggunaan Bahan Sterilisasi dalam Pengendalian Kontaminasi Eksplan tanaman Sirsak (Annona muricata L) secara in vitro* (UNS. Surakarta)

[11] Cahyani R 2018 *Optimasi Inisiasi Dan Variasi Media Penghambat FenolikPada Jati (Tectona Grandis) Secara In Vitro* (Universitas Hasanuddin)

[12] Nasution 2013 *Pengaruh Teknik Sterilisasi terhadap Keberhasilan Inisiasi Eksplan Paulownia (Paulownia elongate SY. Hu) secara In Vitro* (Institut Pertanian Bogor)

[13] Yuliarti N 2010 *Kultur Jaringan Tanaman Skala Rumah Tangga* (Penerbit Andi)

[14] S A Paembonan B B and S H L 2019 Vegetative propagation with branch cuttings as a solution for the mass development of giant atter species (Gigantochloa atter (Hassk.) Kurz) in industrial plantations Vegetative propagation with branch cuttings as a solution for the mass development of *IOP Conf. Ser. Earth Environ. Sci.* 343 012049 343 1–9

[15] Behera S, Kamila P K, Rout K K, Barik D P, Panda P C and Naik S K 2018 An efficient plant regeneration protocol of an industrially important plant, Hedychium coronarium J. Koenig and establishment of genetic & biochemical fidelity of the regenerants *Ind. Crops Prod.* 126 58–68