The optimal sterilizing compound and culture medium in *Elaeocarpus grandiflorus* L. *in vitro* shoot induction

E S Rahayu¹, T Widiatningrum¹, L Herlina¹, N Hermayani² and A R Amalia²

¹ Biology Department Faculty of Mathematics and Science Universitas Negeri, Semarang, Semarang, Indonesia
² Plant Tissue Culture Laboratory, Biology Department Faculty of Mathematics and Science Universitas Negeri Semarang, Semarang, Indonesia

*Corresponding author: enni_sr@mail.unnes.ac.id*

**Abstract.** *Elaeocarpus grandiflorus* L. is an identity plant of Salatiga City. Wherein, the plant is difficult to naturally propagation. This study would obtain propagation technique of *E. grandiflorus* by tissue culture. It was experimentally conducted, consists of two sub-experiments. The first aimed to find the optimal sterilization agent was conducted with a completely randomized design, namely sterilizing compound. There were two compounds studied, i.e., NaOCl and Ca(OC1)₂. The second sub-experiment aimed to determine the optimal medium type and antioxidant compounds for inducing *in vitro* shoots. It was conducted with a completely randomized design with two factors, namely the medium type [i.e. Murashige & Skoog (MS) and Wood Plant Medium (WPM)], and antioxidant agents (i.e. activated charcoal and polyvinyl pyrrolidone/PVP). Data were analyzed by descriptive percentage, analysis of variance, and least significant difference test. The results showed that Ca(OCl)₂ and NaOCl were effective to obtain the sterile and alive explants. The MS media added by PVP resulted in the most explants forming shoots, and WPM supplemented with PVP or active charcoal caused most explants regenerating callus. The results could be used as a basis for the development of *in vitro* shoot multiplication to reach an efficient propagation of *E. grandiflorus*.

**1. Introduction**

*Elaeocarpus grandiflorus* L. was established as identity plant of Salatiga City, Central of Java Province, Indonesia. Wherein, *E. grandiflorus* nowadays is in a very limited number. There are only 20 trees throughout the region of Salatiga, it may be due to the unwillingness to cultivate and the ignorance about the benefits of the plant. In addition, generative propagation through seed germination occurs at a low rate [1]. By considering its efficacy as a medicinal plant and its position as plant identity, it is necessary to find out alternative ways to propagate *E. grandiflorus*. The in vitro propagation could produce true type and healthy seedlings efficiently.

In vitro propagation can be done through various techniques, includes micro-cutting. In principle, the micro-cutting technique is the induction of shoot and root from nodes of the stems. The success of micro-cutting was influenced by several factors especially the effectiveness of surface sterilization, types of culture medium, and the handling of browning symptoms. The first factor aims to prevent contamination that is very common in tissue culture. There are three ways to avoid contamination, namely prevention the existence of microorganisms in explants, prevention the entry of microorganisms
from the air during sub-culture, and reduction contamination at the growth stage of explants in culture bottle. The explant must be surface sterilized to prevent contamination. The surface sterilization includes some techniques depending on the species and plant organs used as explants because each organ has a specific level of contamination. A common way is an application of sterilizing agents. There are various kinds of sterilizing agents used, i.e. sodium hypochlorite (NaOCl), calcium hypochlorite Ca(ClO)₂, mercuric (II) chloride (HgCl₂), silver nitrate (AgNO₃), and hydrogen peroxide (H₂O₂) [2-5].

The second is choice of culture medium types. Among several tissue culture medium recipes, Murashige and Skoog (MS) recipe is most commonly used as a basic medium for induction of callus and shoots. MS medium has high nitrate, potassium and ammonium content and is suitable generally for plant cells growth. However, there is a medium specifically packaged for perennial or woody plants, namely Woody Plant Medium (WPM) which has a low total ion content, but high sulphate and magnesium content to support the growth of woody plant tissue [6].

The third is the handling of browning symptoms. Explant browning is the main impediments in tissue culture. The browning is usually because of phenolic compounds released from the cut surfaces of the explants. The phenolic compounds may not only prevent explant development but also lead to explant death. The oxidation of phenolic compounds could be associated with the sterilization process and specific components of the tissue culture media (e.g., metal cations). The addition of antioxidants into the culture medium could prevent or reduce the oxidation of phenolic compounds. Several antioxidants compound even used namely active charcoal, polyvinylpyrrolidone (PVP), ascorbic acid, and citric acid [7].

The prevention of contamination technique, the type of culture media and browning handling are specific for each plant species. Therefore, it is necessary to find out the most effective factors for certain species. The present study aimed to develop E. grandiflorus shoot induction protocol by optimizing the type sterilizing agent, medium and antioxidant compounds. This study is essential to obtain a large number of sterilized explants and shoots to receive large quantities of E. grandiflorus seedling.

2. Methods

The study was carried out at Plant Tissue Culture Laboratory, Biology Department Universitas Negeri Semarang (UNNES). This study consisted of two stages, namely 1) determination of sterilization agents and 2) determination of medium and antioxidants for explant shoot induction effectively. The explant was stem nodes (2-3 cm) taken from E. grandiflorus trees which were grown at Salatiga City, Central Java Province.

The first stage of the study was conducted experimentally with a completely randomized design of one factor, namely the sterilization agent with multilevel or two concentrations. There are four levels of treatment, i.e. sodium hypochlorite (NaOCl) 15%-10% (N1), NaOCl 20%-15% (N2), calcium hypochlorite [Ca(OCl)₂] 30%-20% (C1) and Ca(OCl)₂ 40%-30% (C2). Five explants were used in each sterilization treatment, and each treatment was replicated eight times. Before being treated, explants were washed and rinsed under water flow for 30 minutes, and then were soaked in 70% ethanol for 1 minute and transferred to the surface sterilization solutions as mentioned above. The explants were soaked for 30 minutes for each concentration of sterilization agent. Each of sterilization solution was supplemented with five drops of tween-20 as wetting agent. After the treatments, all of the explants were washed in autoclaved aquadest three times. The sterilized explants then were inoculated into culture medium containing MS nutrients (Murashige and Skoog, 1962), agar (7g/l), sucrose (30g/l), and 12.3 µM 2iP. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C and 15 bar for 20 minutes.

The cultures were kept in a growth room for one month, at a temperature of 25 ± 2 °C, with a photoperiod of 16 hours, and light intensity of 3000 lux. Contamination rate was determined as the day the contamination began to occur. After one month, the percentage of contaminated explant survived
and dead explants were counted. Data were analyzed by analyses of variance. The differences between
the levels of treatments were considered significant at 0.05 and designated by different letters.

The second stage of the study was arranged with a factorial complete randomized design consisting
of two factors, namely the type of media (MS and WPM) and the type of antioxidant (PVP and activated
charcoal). The four treatment combinations were added by 12.3 μM 2iP. The sterile explants from the
first stage were used in the second stage experiment. Two explants were planted in each combination
treatment, and each was replicated five times. Data observed were rate and percentage of explant
forming shoot, the percentage of explant forming callus and browned explant. The percentage of shoot
and callus formation was determined after 4 weeks. The data were subjected to one-way analysis of
variance (Anova). Data given as percentages were subjected to arcsine transformation before statistical
analysis.

3. Results and Discussion
The results showed that application of sterilizing agents influenced on the percentage of contaminated
and dead explants, but it did not influence on contamination rate. The C1 and C2 treatment (application
of Ca(OCl)\textsubscript{2} 40-30% and 30-20%) could reduce the contamination explant up to 37.50-42.50%.  It was
better than N1 and N2 treatment (NaOCl\textsubscript{2} 20-15% and 15-10%). Among contaminated explants, there
were some recovery explants when transferred to fresh medium, but the others were dead. The highest
number of dead explants occurred in C1 treatment. In all treatments, the emergences of contamination
symptom were similar (Table 1).

Among contaminated explants (Figure 1A) were transferred to a new medium, there were recovery
explants (RE).  The contamination on RE could decrease, lost and the explants become vigor (VE). Like
not-contaminated explants (nCE), the RE capable performed growth and development such as
regenerating callus (Figure 1B) or shoot (Figure 1C). Treatment of N2 resulted in the lowest nCE
(around 20%), but due to high RE, the VE was relatively high (80%) compared to the other treatments.
Explants treated with C1 and C2 have lower recovery capabilities compared to N1 and N2 treatments
(Figure 2).

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treatments (Figure 2).

Figure 1. The growth of E. grandiflorus explant. A. Contaminated explant. B. Callus formed from node
explant. C. Shoot formed from node explant
Table 1. The percentage of contaminated and dead explant, and contamination rate affected by treatments

| Treatment | Sterilization agents (Concentration %) | Contaminated explant (%) | Dead explant (%) | Contamination rate (days) |
|-----------|---------------------------------------|--------------------------|-----------------|--------------------------|
| N1        | NaOCl₂ (20-15)                        | 72.50 b                  | 22.50 b         | 14.50                    |
| N2        | NaOCl₂ (15-10)                        | 80.00 b                  | 20.00 b         | 15.75                    |
| C1        | Ca(OCl)₂ (40-30)                      | 37.50 a                  | 27.50 a         | 13.50                    |
| C2        | Ca(OCl)₂ (30-20)                      | 42.50 a                  | 20.00 b         | 16.50                    |

*Data followed by different letters mean different significance based on LSD 5%.

Based on these results it can be seen that compared to NaOCl, sterilizing agent Ca (OCl)₂ is more effective in suppressing contamination but results in higher death explant and lower recovery ability. As a result the number of competent explants is relatively the same as explants sterilized with NaOCl. The compound of NaOCl and Ca(OCl)₂ are widely used as disinfectants. Ion of hypochlorite (OCl⁻) produced from NaOCl and Ca(OCl)₂ is a strong oxidizing agent. Hypochlorite ions regenerated from hypochlorous acid (HOCI) are dissolved in water and capable to damage bacterial plasma membranes; while hypochlorous acid capable penetrate to the membrane and cell wall of bacteria. Therefore, the two compounds can kill microorganisms. It occurs through inhibition of enzyme activity which essential for growth, membrane destruction and DNA and reduces membrane transport capacity [8].

Figure 2. Percentage of non-contaminated explant (nCE), recovery explant (RE) and vigor explant (VE) affected by treatments of NaOCl₂ 20-15% (N1), NaOCl₂ 15-10% (N2), Ca(OCl)₂ 40-30% (C1) and Ca(OCl)₂ 30-20% (C2)

The results of this study are in line with several other studies. A commercial bleach containing sodium hypochlorite (4.85%) proved to be the most effective for sterilization of seed of *Silphium perfoliatum* L. compared to Ca-hypochlorite, H2O2, and alcohol [2]. Among ethanol, mercuric chloride, ‘flugal’, ‘nystatin’, and sodium hypochlorite, sodium hypochlorite solution application for 30 min was the most effective treatment for *Achyranthes aspera* [4].

The medium type did not influence on shoot emergence rate, the percentage of explants forming shoots and browned explants; on the contrary, it affects the percentage of explants regenerating callus. Explants maintained in WPM media form more calluses than those grown in MS media. Type of antioxidant only changed the rate of explants regenerating shoot, but it does not alter the other three parameters. Explants were grown in medium containing PVP form more shoot than active charcoal. The interaction between types of medium and antioxidants affects all parameters studied. WPM media added by PVP resulted in explants forming shoot faster than the other treatment combinations, and also on the most browned explants. The MS media added by PVP resulted in the most explants forming shoots, and WPM supplemented with PVP or active charcoal caused most explants forming callus (Table 2)
Table 2. The growth of shoots and callus on explants grown in two types of medium and antioxidants

| Parameter                        | Medium type | Antioxidant compound | Mean      |
|----------------------------------|-------------|----------------------|-----------|
| The rate of explant forming shoot (days) | MS          | PVP                  | 27.50 b   |
|                                  | WPM         | Charcoal active      | 32.50 b   |
| Mean                             |             |                      | 30.00     |
| Percentage of explant forming shoot | MS          | PVP                  | 55.00 a   |
|                                  | WPM         | Charcoal active      | 45.00 b   |
| Mean                             |             |                      | 46.25     |
| Percentage of explant forming callus | MS          | PVP                  | 22.50 b   |
|                                  | WPM         | Charcoal active      | 45.00 b   |
| Mean                             |             |                      | 22.50     |
| Percentage of browned explant    | MS          | PVP                  | 22.50 b   |
|                                  | WPM         | Charcoal active      | 37.50 a   |
| Mean                             |             |                      | 30.00     |

* Numbers followed by different letters in the same parameter show significant differences

The E. grandiflorus explants maintained in WPM medium formed more calluses than those grown in MS media. The WPM, which has a low total ion content, but high sulphate and magnesium content, was proven to support the growth of woody plant tissues [6]. This result is in line with other woody plants, such as guava. The highest proliferation rate of guava occurs on the MS medium added by 4.44 μM BA, 4.65 μM kinetin (KT) and 0.54 μM NAA [9].

In the present study, in general, explants grown in MS medium containing PVP formed shoot higher than that in medium containing active charcoal; while supplementation of PVP and active charcoal on WPM caused callus regeneration. This showed that both types of antioxidants were effective in the development of E. grandiflorus explant. PVP is an adsorbent compound that binds to phenols and therefore prevents oxidation. The role of active charcoal includes inactivation of free radicals or the reduction of peroxides, so that unable to form free radicals [7].

The results of this study are in line with the results of other previous studies. Supplementation of PVP, active charcoal and another antioxidant alone or in combination also reduce phenolic oxidation and media browning and therefore increase the capability explant to form callus and shoot of sugarcane [10], guava [11], and Theobroma cacao L.[12].

4. Conclusion
The results showed that both of sterilizing compounds Ca(OCl)₂ and NaOCl were effective to obtain sterile and alive explants. The MS media added by PVP resulted in the most explants forming shoots, and WPM supplemented with PVP or active charcoal caused most explants regenerating callus. The results can be used as a basis for the development of in vitro shoot multiplication to reach an efficient propagation of E. grandiflorus.

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