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The Effect of Various Detergent Concentration and Sterilization Process on Cells from Gametophyte Explants *Acrolejeunea fertilis* (Reinw., Blume & Nees) Schiffn.

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Abstract. Replace *Acrolejeunea fertilis* is a leafy liverwort, part of Lejeuneaceae. The abundance of *A. fertilis* is low hence in vitro culture technique is needed due to their potential as a rich content of terpenoid and flavonoid compounds. As for in vitro culture is a technique for explants multiplication in axenic condition. The aim of this study is to find the optimum detergent concentration and sterilization on the gametophyte explant to achieve viable cell during the in vitro culture hence the used of detergent have never been done in the previous study. Detergent concentration varies in 0.2%; 0.4%; and 0.6% were applied for all the explants during the pre-culture. After that, the explant went through sterilization process using alcohol, disinfectant, fungicide, and antibiotic. Based on microscopic observation, the cell showed an ideal structure after washed using 0.4% detergent compared to 0.2% and 0.6%. The concentration 0.2% indicates the cell is still soiled while 0.6% indicates chlorophyll exuviation. The culture observation lasts for 49 days. In general, the result of this culture has not showed any contamination for the first 21 days but not fully suppress the growth of microorganisms after. The culture showed varies contamination. Contamination from fungi and bacteria occurred on day 22 meanwhile contamination from algae occurred on day 28. Therefore, further development is needed to improve the sterilization methods to obtain axenic conditions with good viable cell conditions.

Keywords: *Acrolejeunea fertilis*, cell, gametophyte, sterilization, tissue culture.

1. Introduction

Acrolejeunea fertilis (Reinw., Blume & Nees) Schiffn. is a species of leafy liverwort classified within the Lejeuneaceae family, specifically the subfamily Ptychantoideae [1,2]. This liverwort is commonly encountered across several Asian countries, including Indonesia, Malaysia, the Philippines, Thailand, India, and Sri Lanka. In Indonesia, its distribution extends across major islands such as Sumatra, Java, Kalimantan, Maluku, and Papua. Ah-Peng et al. (2007) [3] noted that environments characterized by moderate humidity, adequate sunlight, and relatively low rainfall provide favorable conditions for the species, such as those found on the University of Indonesia campus in Depok, West Java. In this area, *A. fertilis* typically grows epiphytically on Bisbul (*Diospyros blancoi*) trees, thriving under microclimatic conditions with light levels between



3409.67 and 6814.22 lux, an average temperature of about 29°C, and approximately 73.17% humidity [4].

Epiphytic mosses provide numerous ecological and biochemical advantages. From an ecological perspective, they function as natural bioindicators, helping to assess atmospheric nitrogen levels and identify air pollutants [5]. These mosses also serve as microhabitats that support diverse microorganisms; liverworts, for instance, are known to harbor various endophytic bacteria, including cyanobacteria [6]. On the biochemical side, liverworts are capable of synthesizing secondary metabolites with antifungal, antibacterial, anticancer, and antitumor activities. Gradstein et al. (2013) [1] reported that *A. fertilis* contains terpenoids and flavonoid-based bioactive compounds. Despite this potential, research on the chemical constituents and applications of *A. fertilis* remains limited, largely because obtaining adequate biomass continues to be a significant challenge.

The cultivation of *A. fertilis* biomass can be supported through in vitro culture methods. This approach relies on the concept of plant totipotency, in which a single somatic cell has the potential to regenerate into an entire organism [7]. In vitro techniques have been widely applied using many plant materials, particularly bryophytes such as mosses and liverworts [8]. For liverwort species, these methods are valuable for examining both morphological and physiological traits. The success of an in vitro culture system is determined by a combination of internal plant conditions, external environmental variables, and the ability to maintain aseptic growth conditions. [9].

Axenic conditions refer to a state in which only the intended explant grows in the culture medium, without contamination from other organisms. Such conditions are maintained through strict aseptic procedures [10]. Several internal variables affect the outcome of in vitro culture, including the kind of explant used, its size and developmental stage, genetic background, and the number of explants placed in the medium [9]. External determinants include the method of sterilizing explants, ambient humidity and temperature, medium pH, the formulation of the culture medium, the addition of plant growth regulators (PGRs), and the level of light provided. Excessive concentrations of sterilizing agents or overly long exposure can injure the explant, whereas insufficient concentrations or short exposure times may fail to eliminate contaminants effectively. The selection of medium components and PGRs must be tailored to the explant type and the specific objectives of the in vitro study [11,12].

Putri (2021) [13] previously conducted an in vitro study using gametophyte explants of *A. fertilis*. Although the explants were able to grow, the cultures showed a high incidence of contamination. A wide range of contaminants was observed, including fungi, bacteria, and algae. In that study, the sterilization protocol involved the use of disinfectants (35% alcohol and 1.25% commercial bleach containing sodium hypochlorite), a fungicide (2% Dithane), and an antibiotic (5% tetracycline). These results suggest that the current sterilant combination still requires refinement. It is proposed that incorporating detergent into the sterilization steps could help reduce contamination levels while keeping the explants viable for subsequent growth. Additionally, using detergent as part of the sterilization treatment may contribute to achieving more reliable axenic conditions, a strategy that has not been tested in earlier research.

2. Methods

The leafy liverwort *Acrolejeunea fertilis* was collected from a *Diospyros blancoi* tree. Several sterilizing agents were used in this study, including detergent at three concentrations (0.2%, 0.4%, and 0.6%), disinfectants (35% alcohol and 1.25% commercial bleach containing sodium hypochlorite), a fungicide (2% Dithane), and an antibiotic (5% tetracycline). All sterilization steps were performed inside a Laminar Air Flow Cabinet (LAFC), except the detergent treatment, which was carried out outside the cabinet. Initially, the explants were rinsed under running water for

three minutes to remove debris and bark fragments. They were then exposed to detergent at the assigned concentrations. Following this, the explants underwent sequential sterilization with 35% alcohol for 30 seconds, 1.25% bleach for 3 minutes, 2% Dithane for 5 minutes, and finally 5% tetracycline for 5 minutes (Figure 1). After each stage, a few lobes were sampled and examined under a light microscope to assess cell integrity—such as the presence of oil bodies, chloroplasts, and other cellular structures—as well as changes in explant coloration. Color assessment was based on Sherwin-Williams color grades. This research relied solely on qualitative observations describing the condition of the explants after each sterilization step.

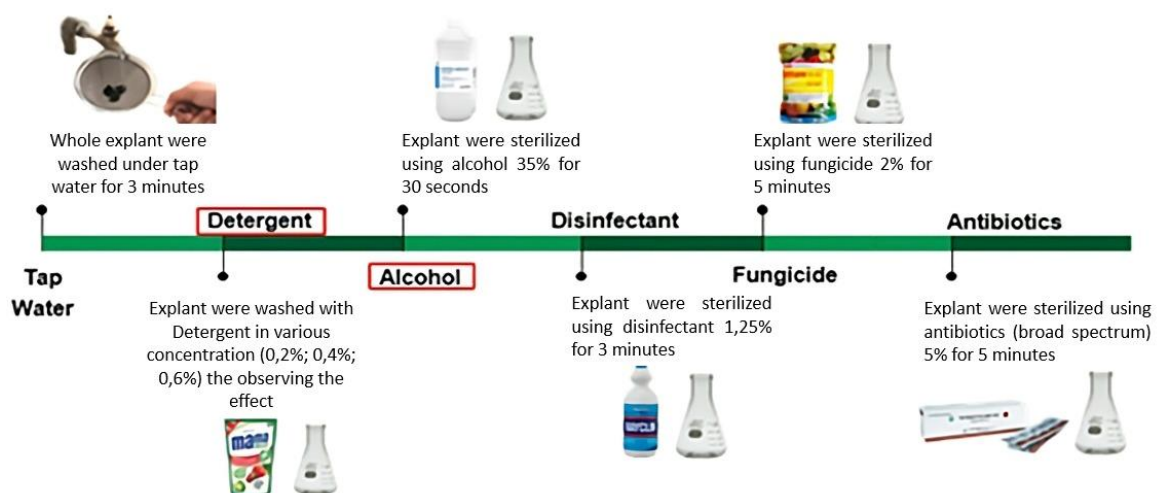


Figure 1. Flow Chart of *A.fertilis* Sterilization Proces (Putri 2021 [13] with modification)

3. Results and Discussion

The coloration of the explants was examined at multiple points: immediately after removal from the host tree (before rinsing with distilled water), after treatment with the different detergent concentrations, and again following the full sterilization sequence—which included exposure to 35% alcohol, 1.25% sodium hypochlorite, 2% fungicide, and 5% antibiotic. As shown in Figure 2, the initial coloration based on the Sherwin-Williams chart matched the shade “Paradise” (Figure 2A). Following the detergent wash treatments, the explants generally retained the same color category; however, cellular impurities became visible in those treated with 0.2% detergent. At the highest concentration (0.6%), cells appeared empty and structural rupture was evident. In contrast, explants washed with 0.4% detergent maintained intact cell compartments and displayed no observable impurities, suggesting that this concentration did not alter pigmentation or compromise cellular structure.

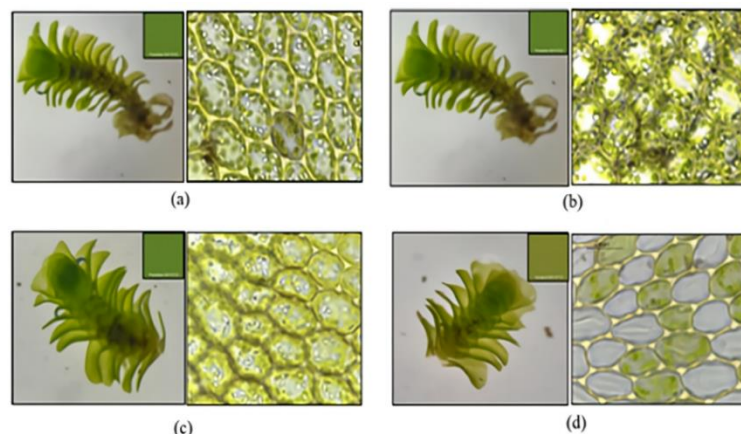


Figure 2. Explants color and cell structure after treatments (under light microscope, magnification 1000x). (a) aquadest; (b) Detergent 0,2%; (c) Detergent 0,4%; (d) Detergent 0.6%

Microscopic examination showed that explants treated with 0.4% detergent retained healthy cell conditions. The chloroplasts, oil bodies, and firm trigones were still present, and no changes in coloration were detected. However, once the explants were subsequently exposed to the remaining sterilants 35% alcohol, 1.25% commercial bleach, 2% fungicide, and 5% antibiotic clear alterations in both cell structure and pigmentation appeared. Alcohol, in particular, caused noticeable browning of the tissue (Fig. 3A). As a hypertonic and phytotoxic agent, alcohol can compromise membrane integrity, dehydrate cells, induce stress responses, and trigger the formation of phenolic compounds, which result in tissue browning. Explants with more delicate structures are especially vulnerable to this damage.

Before alcohol exposure, the cells displayed well-defined hexagonal outlines, firm trigones, and chloroplasts and oil bodies distributed throughout the cytoplasm. After 30 seconds in 35% alcohol, these features changed significantly. The chloroplasts, which had previously been dispersed, appeared to cluster near the cell membrane, suggesting the onset of plasmolytic activity (Fig. 3B). This occurs when water exits the cell due to a higher solute concentration in the external solution. Nevertheless, based on Figure 3B, the cells had not reached full plasmolysis; instead, they showed only cytoplasmic contraction rather than complete detachment of the plasma membrane.

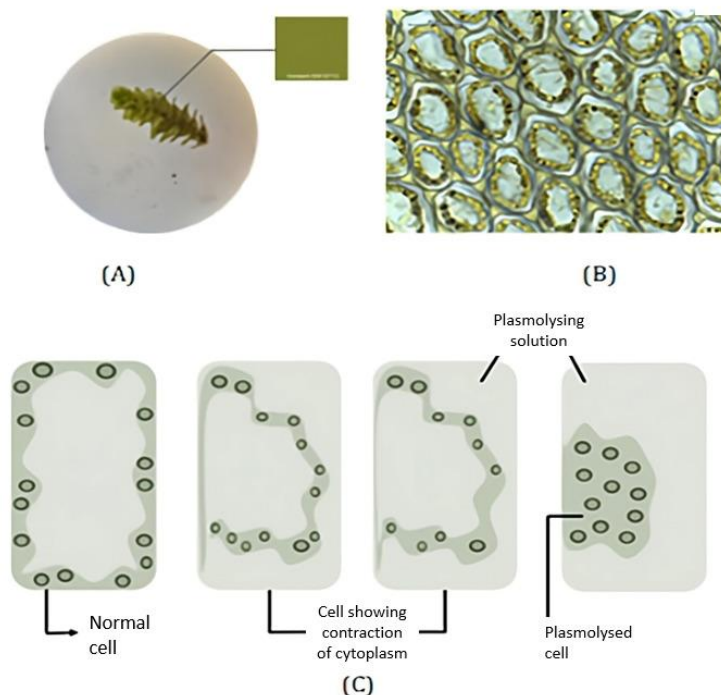


Figure 3. (A) Explant's Condition after Sterilization using Alcohol 35% Macroscopic Condition; (B) Cell's Observation under Light Microscope (Magnification 1000X); (C) Illustration of Plasmolysis Mechanism

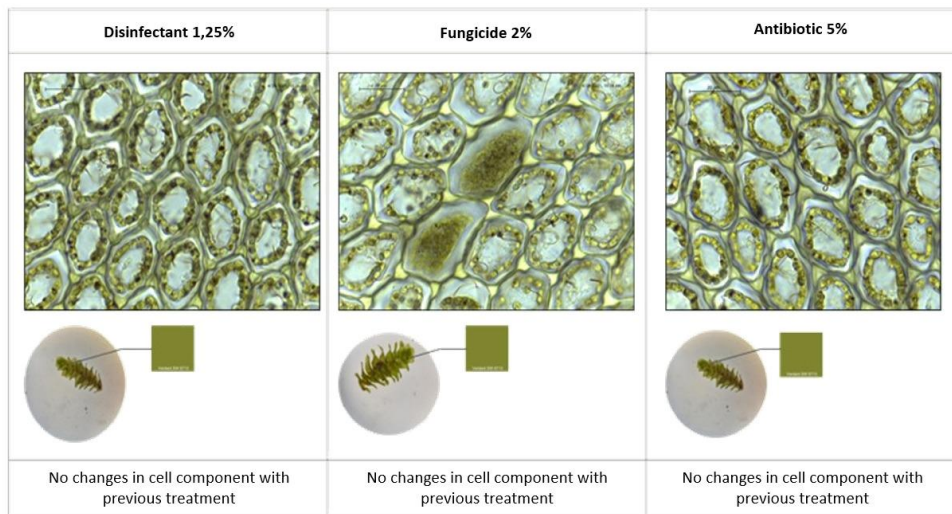


Figure 4. Explant's Cell Condition after Sterilization using Disinfectant, Fungicide, and Antibiotics Observed under Light Microscope (Magnification 1000X)

The darkening of the explants following sterilization may also be attributed to the use of commercial bleach (sodium hypochlorite/NaOCl). Putri (2021) [13] reported that exposure to 1.50% commercial bleach can lead to immediate browning of plant tissues. This discoloration often results in reduced explant viability and may even lead to tissue death [14]. When explants are treated with high concentrations of sodium hypochlorite, they experience stress that stimulates the production of phenolic compounds. These polyphenols are responsible for tissue browning because they activate polyphenol oxidase, which converts them into quinones through

oxidation. Quinones can then diffuse into the surrounding medium, inhibit enzymatic activity, and render the culture environment toxic to the explants. Prolonged exposure may ultimately kill the tissue [15,16]. Antioxidants such as polyvinyl pyrrolidone (PVP) or activated charcoal can help reduce this oxidation and limit browning [16]. Despite the discoloration, the explants treated sequentially with 1.25% disinfectant, 2% fungicide, and 5% antibiotic did not exhibit any notable microscopic structural changes (Figure 4)

4. Conclusions

The optimum detergent concentration for *Acrolejeunea fertilis* sterilization is 0.4%. Exposure of alcohol 35% for 30 seconds may be destructive to *A.fertilis* cell, browning condition, and cause growth inhibition during *in vitro* culture. Further studies are needed to ensure the optimum concentration and exposure time for sterilization using alcohol that will not affect the explant cell's condition.

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