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OPTIMIZATION OF PROTOPLAST ISOLATION FROM DIFFERENT TYPES OF SAMPLES

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ABSTRACT

Isolation of protoplasts in several plant species still requires optimization to produce protoplasts that are viable and can be used for further analysis. This research aims to optimize protoplast isolation protocols in several plants: Orchid *Dendrobium macrocarpus*, potato *Solanum tuberosum*, and microalgae *Phaeodactylum tricornutum*, *Botryococcus braunii*, and *Spirulina* sp. The results showed that *in vitro* explant selection had higher sample uniformity, and a combination of enzyme solutions could be used to increase the effectiveness of protoplast isolation on *D. macrocarpus*, *S. tuberosum*, and *Spirulina* sp. This study provides information about protoplast isolation techniques and testing their viability as an attempt for plant breeding through micropropagation.

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INTRODUCTION

Protoplast culture is a method that induces the emergence of variation within the plant itself, by encouraging the expression of genetic traits that are usually hidden or do not appear in the phenotype (Reed & Bargmann, 2021). This technique develops from the principle of cell totipotency, where each cell has the ability to grow and develop in a suitable environment, carrying its own independent character. By isolating each cell from the plant and regenerating it in a suitable medium, new plants will be obtained that carry the characteristics of these cells (Charrier et al., 2019).

In carrying out protoplast fusion, protoplast isolation is first carried out. Protoplasts can be isolated from almost all plant parts, such as roots, leaves, root nodules, coleoptiles, callus cultures and in vitro leaves (Bertini et al., 2019). Protoplasts can be isolated mechanically or enzymatically. Enzymatically, the type and concentration of enzyme used greatly influences the yield of protoplasts. Young cell walls are usually composed of pectin and cellulose. Therefore, the most suitable enzyme to use is Pectinase or Macerozyme and Cellulase (Lopez-Arellano et al., 2015).

Potatoes are a popular vegetable and food crop because they have a good taste, balanced nutrition, the price is quite high, the tubers are relatively not easily damaged and price fluctuations are low (Moon et al., 2021). However, potato plants are also known as plants that are very susceptible to pests, diseases and other environmental stresses (Das et al., 2000). Introducing disease resistance genes into potato plants can be done using the protoplast fusion method. Menke et al., (1996) stated that the plant leaf mesophyll that is cultured in vitro and is most widely used as a source of protoplasts is Solanaceae. Several studies have been carried out on protoplast fusion in Solanaceae, including hybridization of Solanum malmeanum with S. tubesorum to produce frost-tolerant hybrids (Tu et al., 2021). Sihachakr et al., (1989)have succeeded in producing somatic hybrids from Solanum melongena with Solanum khasianum using the electrofusion method.

Apart from potatoes, somatic hybridization is also carried out on orchids. Currently there are a number of studies on protoplast culture in several orchid genera, such as Cymbidium (Ren et al., 2020), Dendrobium (Kanchanapoom, 2001), and Phalaenopsis (Li et al., 2018). Among these orchid genera, Dendrobium is one of the most popular orchid genera and is developed to obtain somatic hybrids. Several Dendrobium cultivars and hybrids originate from conventional methods and somaclonal variation. Even though there are many intrageneric and intraspecific Dendrobium hybrids, it is very difficult to produce intergeneric hybrids using conventional breeding techniques, so somatic hybridization can be carried out through protoplast fusion to combine and hybridize Dendrobium species that are sexually incompatible (Ren et al., 2021).

MATERIALS AND METHOD

Materials

The plant materials used in this research consisted of plant materials and chemicals. Plant materials are in vitro Dendrobium macrocarpus orchid plantlets, in vitro potato plantlets, Phaeodactylum tricornutum, Botryococcus braunii, and Spirulina sp. Meanwhile, the chemicals used are sucrose solution, components that make up enzyme solutions (Table 1), components that make up CPW solutions (Table 2), and fluorescein diacetate (FDA).

Table 1. Components of enzyme solutions

Components	Volume
Cellulase Onozuka R-10	1,2% w/v
Macerozyme R-10	0,4% w/v
Mannitol	13% w/v
Dissolved in CPW solution and sterilized with a Millipore filter, pH 5.8	

Table 2. Components of CPW solutions

Components	Volume
KH2PO4	27,2 mg/l
KNO3	101 mg/l
CaCl22H2O	1480 mg/l
KJ	0,16 mg/l
CuSO45H2O	0,025 mg/l

Methods

1. Removal of cell walls

The process of removing cell walls is divided into two, namely in microalgae, and orchids and potatoes. In microalgae, 3 cc of enzyme solution was added each to the suspension of *P. tricornutum*, *B. braunii*, and *Spirulina* sp. separately, then incubated at 25ºC for 18 hours. For orchids and potatoes, this is done by slicing each potato and orchid leaf to a length of ± 3 cm using a sharp scalpel. Into the petridish containing the leaf slices, add 5 cc of enzyme solution or until it has soaked all the leaf slices. Then incubate at 25ºC for 18 hours.

2. Microscopic observation

Eighteen hours after incubation, observations were made using a microscope to determine the protoplasts and orchids and potatoes that were formed as a result of the previous enzyme treatment.

3. Protoplast washing

Each microalga was taken and put into a test tube using a Pasteur pipette. Meanwhile, for orchids and potatoes, the solution was taken using a Pasteur pipette and filtered into a test tube using a nylon filter. The filtered microalgae, potato and orchid protoplasts were collected in a centrifuge tube and then centrifuged at a speed of 1000 rpm for 10 minutes.

The supernatant was removed using a Pasteur pipette and the washing solution was slowly added into the tube. The solution was resuspended carefully until homogeneous. The protoplast suspension was then centrifuged at 1000 rpm for 10 minutes, then with a Pasteur pipette the supernatant was discarded.

4. Purification of protoplasts

Three ml of sucrose solution were added with the pipette tip to the bottom of the tube. The protoplast suspension will float on the surface. The suspension is then centrifuged at 1000 rpm for 10 minutes, the protoplasts will be separated from the debris. Pure protoplasts are in the top layer while debris is at the bottom of the tube. Using a pipette, the pure protoplast suspension on the surface was taken and viability tested by painting with fluoroscein diacetate (FDA). The protoplast suspension was placed on a glass slide and dripped with FDA, then observed with a fluorescein microscope.

RESULTS AND DISCUSSION

This study used five *in vitro* samples. Sample selection is very important in the protoplast isolation process. Menke et al., (1996)stated that the best protoplast samples to use are *in vitro* leaves. This is because the physiological condition of plant leaved from *in vitro* culture is more constant than from the greenhouse. Moreover, *in vitro* leaf uniformity is higher and can be available at any time and does not require sterilization.

The enzyme treatment was treated the same for microalgae, potato, and orchid. The objective of enzyme treatment is to temporarily remove cell walls. As a mechanical support for plant tissue, cell walls are very complex and highly differentiated. The process of removing cell walls to obtain protoplasts can be done mechanically or enzymatically (Jia et al., 2018). The mechanical method is done by cutting the sample in a plasmolytic solution. The protoplast will shrink, so that it can be pressed out of the cell wall. Deplasmolysis will then cause the release of protoplasts from the cells. The disadvantages of using this technique are that it is relatively difficult, the number of protoplasts produced is not large, its effectiveness is limited only to cells that can be plasmolyzed such storage tissue and cannot be used on meristem tissue because the cell walls are still very closely connected to the protoplast. Its advantages can negate the effects of enzyme activity which sometimes damages or disrupts very complex metabolism in protoplasts (Li et al., 2018; Ren et al., 2021).

Enzymatic isolation of protoplasts using enzyme solutions with various compositions. To properly lyse cell walls, this can be done by using a combination of two types of enzymes, cellulase and pectinase simultaneously. Pectinase will loosen the bonds between one cell and another or release cells, while cellulase will destroy the cellulose wall (Yao et al., 2016). The enzyme solution used in this research consists of a combination of cellulase and macrozyme enzymes. Several studies have used a combination of enzymes. Larkin, (1976)used cellulase Onozuka P1500 3% and macerozyme 0.25% to isolate nicotiana leaf protoplasts and petunia flower jewelry. (Hahne et al., 1983) used an enzyme solution consisting of cellulase (Roem, Darmstadt) 1%, pectinase (PATE, Hoeschst) 0.1%, macerozyme R-10 0.1% and mannitol 0.4 M at pH 5.8. Cellulase enzymes that are often used are driselase, cellulisin, and cellulase Onozuka R-10. Meanwhile, other enzymes that are often used with cellulase are hemicellulose (Rhozyme HP 150) and pectinase (macerase, macerozyme, pectiol AC, pectolyse Y-23, pectinase, peptic acid acetic transferase).

The enzymes used for protoplast isolation are products of several types of microorganisms (Sun et al., 2018). These enzymes are generally traded with different levels of purity, this shows that are still other components in them, for examples, cellulase may also contain hemicellulose (Wang et al., 2015). The availability of impure

enzymes is actually also beneficial because it can hydrolyze cell wall components that are not substrates for the main enzyme. The Onozuka R-10 cellulase enzyme, for example, is known to contain hemicellulose, this enzyme is most often used for isolating protoplasts from various types of plants. Enzyme sterilization is carried out using filters with a porosity of 0.22 – 0.24 µm, because enzymes are thermolabile. The filter is placed at the end of the injection tool, the enzyme solution is passed through the filter, so that the solution that comes out is a sterile enzyme solution.

Eighteen hours after incubation, microscopic observations were carried out in orchid, potato, and microalgae protoplasts. From the results of observations using a microscope, it can be seen that there are intact or round, intact and viable orchid and potato protoplasts with transparent and clear membranes, so that the organelles or insude of the cells are clearly visible (Figures 1a – 1b). the most clearly visible organelle is chlorophyll which shows green granules in relatively large numbers and relatively large in size. Therefore, chlorophyll is used as a marker in identifying the yield of isolated protoplasts (Zhang et al., 2011). In *B. braunii* it cannot be known wether the protoplasts has successfullt formed or not, because the cell shape is also round (Figure 1c). Similar with *P. tricornutum*, the isolated protoplasts could not be properly observed (Figured 1d). however, in *Spirulina* sp. several protoplasts has successfully formed (Figure 1e). This is due to the cell shape of *Spirulina* sp. which is oval in shape, so they can differentiate easily.

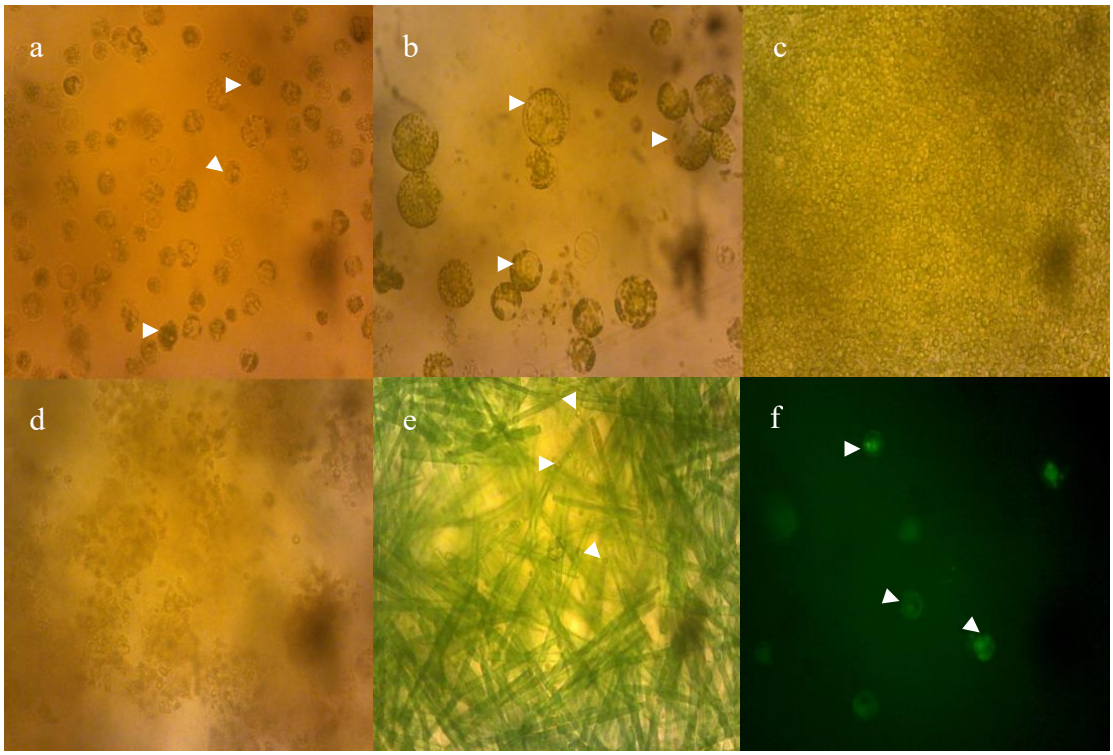

Figure 1. Isolation results of orchid protoplasts (a), potato (b), *B. braunii* (c), *P. tricornutum* (d), *Spirulina* sp. (e), and FDA staining of orchid protoplasts (f). Arrow head indicates successfully formed protoplasts.

After incubation, the protoplasts were washed to remove the effect of enzyme treatment. In order to obtain intact protoplasts, a sucrose gradient treatment was carried out by adding 3 ml of surose solution to the protoplast suspension (Figure 2a). After centrifugation, sucrose with a certain molecular weight will be able to precipitate debris in the form of remnants of epidermal tissue, transport tissue, damaged protoplasts and cell aggregates, while viable protoplasts will float on the surface of the solution (Figure 2b).

The next stage of the protoplasts isolation process is staining with flouroscein diacetate (FDA) to determine protoplast viability. Viable cells will produce a green color when observed under a fluorescence microscope (Shao et al., 2023). The protoplast used for staining was only orchid protoplast, because too little protoplast was produce from potatoes and microalgae. The observation results showed that only a few orchid protoplasts emit green color under the microscope (Figure 1f). The staining process can also use calcofluor white (CW) solution (for cell wall regeneration) or FDA double staining with propidium iodide (PI). The first two fluorescein stains

can only color viable protoplasts, because the paint can only collect in the plasmalemma of viable protoplasts, which can be detected with a fluorescence microscope. PI can stain dead cells, with double staining of live and dead protoplasts can be detected. Doods and Roberts (1983) used Evan's blue 0.1% to test protoplast viability. Viable protoplasts will be able to reject the biological dyes. Cell impermeability to these paints can be used as an indicator of protoplasts viability.

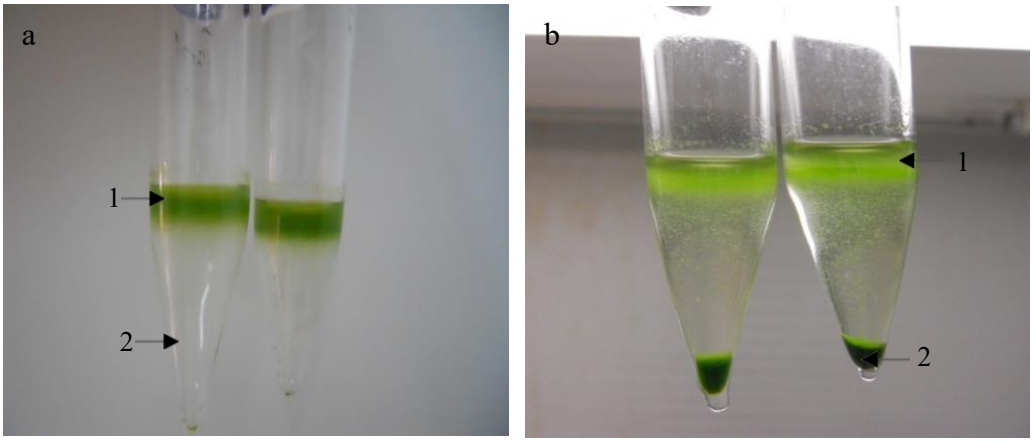

Figure 2. Addition of sucrose solution to protoplast suspension (a): Mixture of protoplast suspension (1), sucrose solution (2). Centrifugation results (b): Pure protoplasts (1), debris (2).

CONCLUSION

Protoplasts of *D. macrocarpus*, *S. tuberosum*, and *Spirulina* sp. can be isolated using a combination of cellulase enzyme solution and macerozyme enzyme. The results of isolated protoplasts in B. braunii and P. tricornutum could not be observed properly because the cells were round in shape, so they could not be distinguished from isolated protoplasts. The most protoplasts that were successfully isolated were orchid protoplasts and then continued with FDA staining. The staining results showed that several protoplasts glowed green under a fluorescence microscope, indicating a viable protoplast population.

AUTHORS CONTRIBUTION

W.M. designed and conducted the research, analysed and interpretation the data, and wrote the draft of manuscript, S.W. analysed and intrepetation the data.

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