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Research Article

Establishment of A Transient Expression Using PEG-Mediated Protoplast Transformation System in Black Rice *Cempo Ireng*

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Abstract
Indonesia black rice is a potential crop which consider to be develop as functional food because of high nutritional values. However, some agronomical traits, such as high culm and long harvesting time need to be improved for high productivity. Genetic engineering based on protoplast system is one of tools that can be used for improving black rice agronomical traits. The purpose of this study was to establish an efficient method for obtaining protoplasts, and to get information on whether the PEG-mediated transformation method can be carried out on black rice '*Cempo Ireng'* using GFP transient expression as a marker. To get protoplast culture, we used callus and seedling as main explants. The results showed that 15th days seedling was the best explant source to get protoplast compare to callus. The combination of 1,5% macerozyme and 3% celulase was optimum to obtain the viable protoplasts. Transient expression of GFP can be done using PEG-mediated protoplast transformation in 30% concentration of PEG.
Keywords*: Black Rice; Cempo Ireng; Transformation; PEG.*

Introduction

Black Rice (*Oryza sativa* L. cv. *Cempo Ireng*) is rice that has a high nutrient content such as high fiber, iron, amino acids and anthocyanin. Extract black rice is rich in anthocyanins that identified containing cyaniding-3-glucoside and peonidin-3-glucoside. Anthocyanin extract is reported to improve lipid profiles in apolipoproteinEdeficient mice by lowering levels of triglycerides, total cholesterol and non-HDL cholesterol [1], [2].

Black rice productivity, however, is low due to its long harvesting time, caused by late heading date, and therefore poor yield. These negative traits in black rice may be improved using conventional and modern approaches by manipulating genes of interest [3].

Purwestri [1], have tried to shorten the harvest period by introducing a rice flowering gene, *Hd3a,* under the *rolC* phloem-specific promoter [4], but they failed to regenerate plantlets from calli. Regeneration from calli through somatic embryogenesis has been achieved in temperate japonica rice [5]. The established methods for temperate japonica rice were adopted in previous study to regenerate plants from calli. However, the genotypes used in different tissue culture methods yielded mixed results [3], [6], [7]. Reported that each genotype of javanica rice needed an optimum medium and optimization of the plant tissue culture method [8], [9]. The establishment of an appropriate plant tissue culture regime is therefore essential to support black rice cultivar

improvement through various plant biotechnological methods, including applying transgenic approaches [3]. Here, we described a method for the isolation and transformation of protoplasts from the rice cultivar *Cempo Ireng* Wild Type. The method has been developed based on previous protoplast transformation protocols [10].

Materials and methods

Seed of black rice cultivar *Cempo Ireng* Wild Type used in this study was collected from Research Center for Biotechnology, Universitas Gadjah Mada.

1. *Black rice seed surface sterilization, callus and seedling induction.*

The scutellum was considered as the best explant for rice tissue culture [3]. Mature seeds of black rice were dehusked manually and sterilized by immersion in 70% (v/v) ethanol for 1 min and then in 5.25% sodium hypochlorite for 2 minutes. They were then washed 3 times for 5 min each with sterile water. The sterile seeds were dried on sterile filter paper and cultured with the scutellum pointing upwards on callus induction medium that are listed in Table 1.

Table 1. Culture media

Culture medium	Purpose	Composition for 1:1
MS	Seedling induction	4.2 g MS powder, 20 g sucrose, 0.1 g myo-inositol, 2 mg glycine, 0.5 mg thiamine, 0.5 mg nicotinic acid, 0.5 mg pirydoxine, 8 g agarose, 0.2 mg zeatin, 1 mg NAA (pH 5.6 to 5.8)
2N6	Callus induction	4 g N6 powder, 30 g sucrose, 1 mg thiamine, 0.5 mg pyridoxine, 8 g agarose, 4 mg 2,4-D (pH 5.6 to 5,8)
LB	*Escherichia coli* culture	Tryptone 10 g, yeast extract 5 g, NaCl 10 g, Bacto agar 15 g (pH 5.7)

2. *In vitro genetic transformation*

Escherichia coli strain *DH5α* was cultured on LB liquid medium for 24h at 37 ºC in the dark. *Escherichia coli* starter culture was suspended in 200ml LB medium and incubated on shaker at 250 rpm and 37 ºC in the dark for 3h until OD_{600}. the culture was then placed in ice for 30 minutes The next stage, the culture was divided into 4 50 ml conical tubes in ice and centrifuged at a speed of 4000 rpm at 4°C for 15 minutes. The supernatant obtained was then discarded and the pellets were resuspended with 5 ml of cold MgCl2. After that it was centrifuged at a speed of 3000 rpm at 4°C for 15 minutes. The result of centrifugation in the form of a supernatant was discarded and the pellets were resuspended with 2.5 ml of cold CaCl2 then incubated in ice for 20 minutes. Resuspension results were centrifuged at a speed of 3000 rpm, at 4°C for 15 minutes. The supernatant obtained was discarded and the pellets were resuspended with a mixture of 1.25ml 85 mM CaCl2 + 15% cold glycerol solution. The resuspension product was centrifuged at 2100 rpm at 4°C for 15 minutes, and the supernatant was discarded. The pellets obtained were resuspended with 0.5 ml 85 mM CaCl2 + 15% cold glycerol. The results of the resuspension were aliquoted into 1.5 ml microtubes each of 50 µl. The cultures were frozen with liquid nitrogen for storage at -80°C.

3. *Isolation of protoplast*

Sixty seedlings were harvested by cutting the base of the plant at medium level. Approximately 60 mm of stem and sheath tissue was retained (Figure 1) and briefly rinsed with water to remove any adhered compost. The plant tissue was cut into 0.5 mm using a sharp blade, directly into 20 ml 0,6 M mannitol inside an erlenmeyer flask. The tissue was incubated in the dark for 10 min at room temperature (RT) to initiate plamolysis. After discarding the mannitol, the strips were incubated in an enzyme solution (3% celulase RS, 0.75% macerozyme R-10, 0,6 M mannitol, 10 mM MES at pH 5.7, 10 mM $CaCl_2$, and 0,1% BSA) for 4-5 h in the dark with gentle shaking

(50-70 rpm) to allow digestion of cell wall material. W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5mM KCl and 2mM MES at pH 5.7) was added and the flask shaken gently by hand for 10 s to terminate digestion. Protoplasts were released by filtering through 40 µm mesh by gravity to release protoplasts, and the retained tissue rinsed with an additional 20 ml W5 solution for 3-5 times. Then centrifuged for 3 minutes at a speed of 1500 rpm. The resulting pellets were then rinsed with W5 buffer dam resuspended with ± 100 µl of MMG solution.

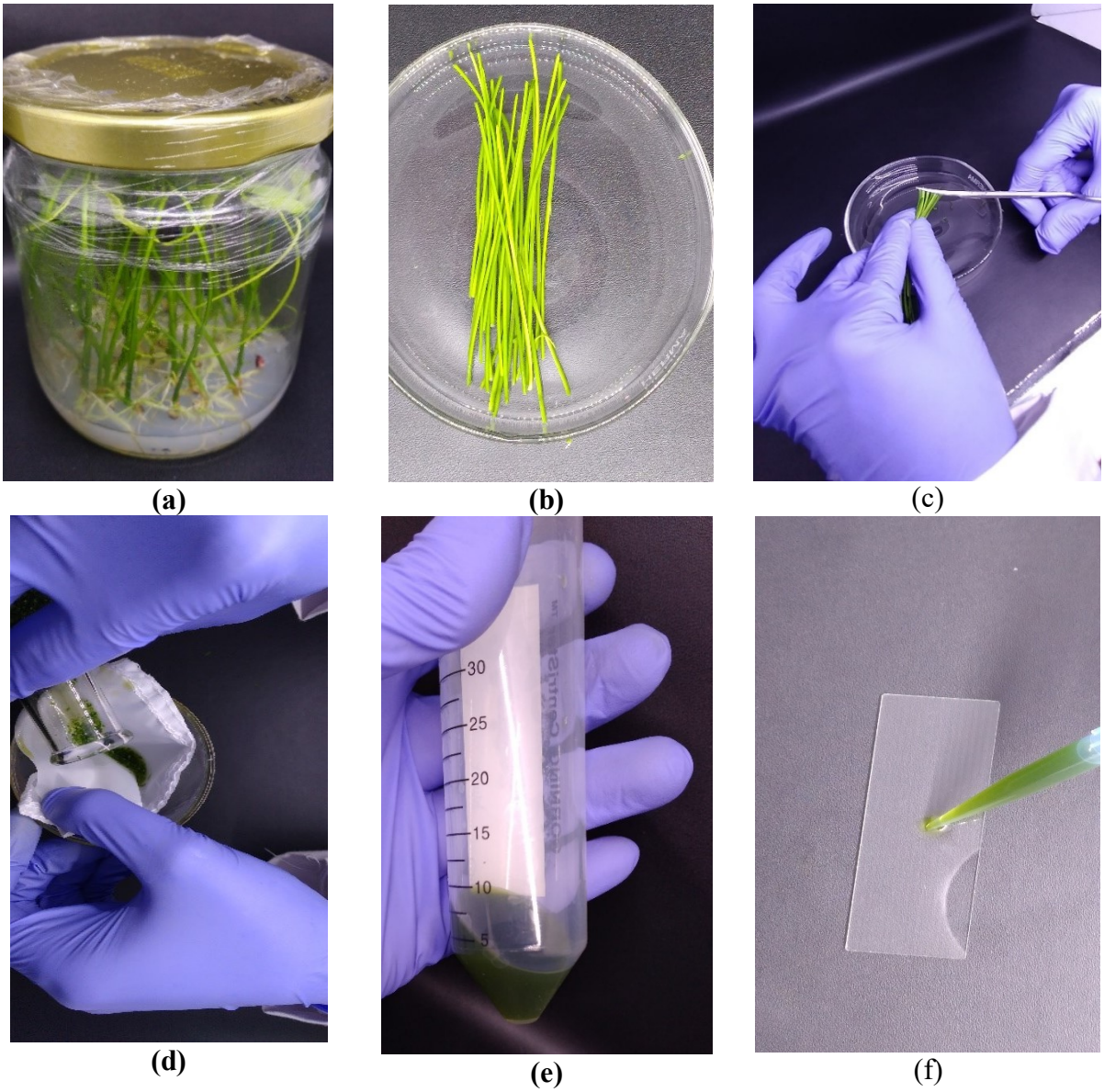


Figure 1. Photographs of various steps in the protocol. (a) Seedling of black rice *Cempo Ireng* Wild Type 15 day-old in MS medium, (b) approximately 60 mm of rice stem and sheath tissue, (c) cutting tissue into mannitol to initiate plamolysis, (d) filtration of digested tissue through 40 µm mesh, (e) protoplast suspension after isolation, (f) observation of protoplasts.

4. *Transformation of protoplasts*

5µl plasmid DNA were mixed with 50 µl protoplasts in 1.5 mL microsentrifuge tube. 110 µl freshly PEG solution [30% (W/V) PEG6000; 0,2 M mannitol, and 0,1M $CaCl_2$] were added, and the mixture was incubated at RT for 10-20 min in the dark. The transformation process was terminated by addition of 440 µl W5 solution (0,5 M mannitol, 20 mM KCl, and 4 mM MES at Ph 5.7). Finally, protoplasts were transferred into multi-well plates and cultured under light or dark at RT for 6-16 h.

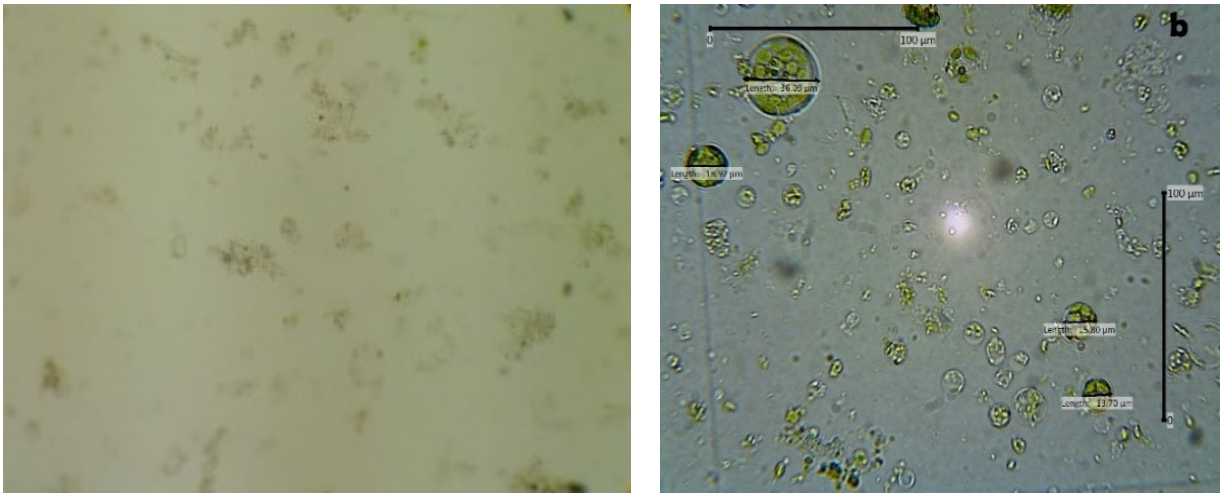
5. Data analysis

Data from protoplast isolation were analyzed by descriptive analysis. The results of the observations were in the form of optimization data for each isolation stage of protoplasts and protoplasts which can be transformed by plasmids. The results of the protoplasts can be clearly seen under a microscope. Other results observed were screened live cells carrying the transformed GFP gene, which were observed under a confocal microscope.

Results and Discussion

1. Protoplasts isolation and transformation efficiency

We used 2 material explants to optimize, there were calli and seedlings. The viable callus were cut and transferred to the erlenmeyer and then added with 2N6 liquid media then shaken for ± 1 month. The results of the treatment were not optimal because the callus became contaminated or died. The breakdown of callus into cells is difficult to do even after being shaken for 3 months. Then another treatment was carried out, callus that were ± 3 weeks-old were cut into small pieces and added with enzyme solutions. When observed under a microscope, we didn't find callus cells. It can be concluded that the results of protoplast isolation with black rice callus *Cempo Ireng* Wild Type did not show maximum results (Figure 2). Beside bad results, isolation with callus took longer time because the viable callus used was ± 2-3 weeks of age compared to seedling which took 7-15 days. According to Zhang, there are several methods that can be used to produce protoplasts [10]. They can use callus, leaves, and seedling. Using callus is less efficient and relies on expensive tools. The use of leaves is also considered less efficient.



(a) **(b)**

Figure 2. Figure of protoplasts isolation from (a) callus, (b) seedlings of black rice *Cempo Ireng*

2. Optimization of seedlings' age for protoplasts isolation

A good protoplast is an essential condition for successful transformation and analysis. It is very important to prepare protoplasts from fresh and good seedlings. Said that fresh and tender rice seedlings were key to isolating protoplasts that could be transfected at high efficiencies, with 7-10 days-old seedlings being the most suitable for this purpose [10]. The transfection efficiencies were variable in protoplasts from 11 to 14-day-old seedlings and declined sharply with those from seedlings older than 14 days. In contrast, other reported methods utilize 2-week-old or 2-month-old rice green tissue, antibiotics, toxic chemicals or vacuum.

Based on the table, the most protoplasts were produced from 11 days-old seedlings with $15{,}500 \times 10^3$ cells, but the protoplasts could not survive after 72 hours. Only protoplasts from 14 and 15 days-old seedlings were still survived after 72 hours. Based on Zhang the protoplasts required for transfection of at least 2×10^5 cells, the protoplasts from 15 day-old seedlings are

considered sufficient for transfection needs with $127x10^5$ cells, so for the next stage optimizations we used 15-day-old seedlings [10].

Table 1. Optimization of seedling age (with concentration of maserozyme 1.5% and celulase RS 3%)

No	Seedlings' age	Amount of protoplas	After 3 h	After 18 h	After 72 h
1.	7 old days	$5.340x10^3$	$2.600x10^3$	-	-
2.	8 old days	$6.880x10^3$	$4.850x10^3$	-	-
3.	9 old days	$8.200x10^3$	$5.670x10^3$	-	-
4.	10 old days	$8.860x10^3$	$6.980x10^3$	5	-
5.	**11 old days**	**$15.500x10^3$**	**$10.920x10^3$**	**25**	**-**
6.	12 old days	$13.800x10^3$	$10.100x10^3$	10	-
7.	13 old days	$11.200x10^3$	$8.600x10^3$	24	-
8.	14 old days	$11.060x10^3$	$7.700x10^3$	26	5
9.	**15 old days**	**$12.760x10^3$**	**$9.260x10^3$**	**78**	**13**

3. Optimization of enzyme solution's concentration

Enzyme solution is the most important component to produce protoplasts. The composition of the appropriate enzyme solution greatly affects the success rate of protoplast isolation. Each type of plant requires a different concentration of enzyme solutions to get maximum protoplast results [11]. Suggested that it is better to use a combination of the Cellulase enzyme with the Pectinase enzyme such as Macerozyme for the protoplast isolation process [12]. The combination of these enzymes will increase the density of isolated protoplasts.

Table 2. Optimization of enzyme concentration (with 60 seedlings)

No	Enzyme concentration	Amount of protoplasts
1.	Maserozyme 0,75 % Selulase RS 1,5%	$6.540x10^3$
2.	**Maserozyme 1,5%** **Selulase RS 3%**	**$14.200x10^3$**
3.	Maserozyme 2% Selulase RS 4%	$8.900x10^3$

The use of 1% cellulase from wild rice leaves did not produce protoplasts, while the use of 2% cellulases only produced protoplasts with a very low density. The result was that enzyme solution with maserozyme 1.5% and cellulase RS 3% produced more protoplasts with $14.200x10^3$ cells. There was a degradation when using enzyme solution with maserozyme 2% and celulase RS 3%. Said that increasing enzyme concentration can increase protoplasts, but excess enzyme concentration can reduce the amount of protoplasts and their viability [12], [13]. This is possible due to the phytotoxicity of the enzymes on the protoplast membrane.

4. Optimization of centrifuge

Centrifugation is one of the steps to separate viable protoplasts from their debris. At this stage the centrifuge speed greatly affects the density of the protoplast because of the fragile and fragile nature of the protoplast. Protoplasts are very vulnerable after being inserted into a liquid so they must be treated gently. Protoplasts can rupture due to the high centrifuge speed, a speed of 1000 rpm is considered safe for most protoplasts [4].

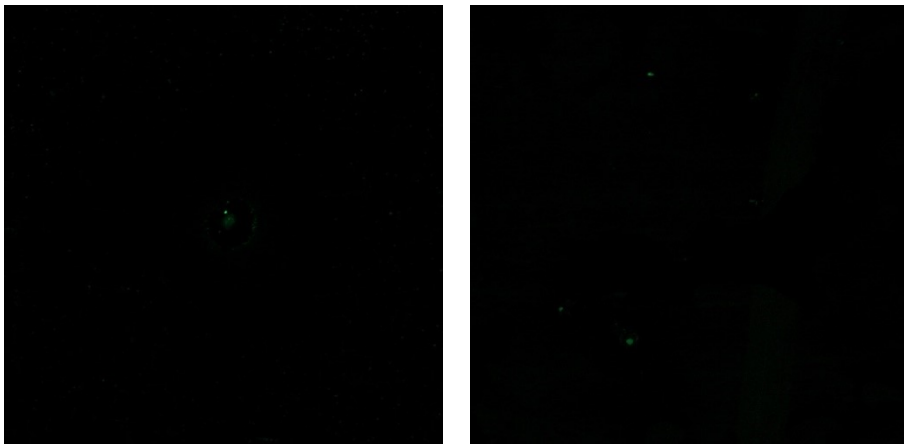
Table 3. Comparison of the amount of centrifuge and non-centrifuge protoplasts. With composition of enzyme solution is maserozim 1,5% and cellulase RS 3%; 15 day-old plant.

Non-centriguge	$15.500x10^3$
Centrifuge 700rpm	$7.200x10^3$

In this study there was a decrease in protoplast density which was quite high when centrifuged, from $15,500x10^3$ protoplasts/mg to $7,200x10^3$ protoplasts/mg (Table 3). This could be due to the centrifuge speed that was too high or the condition of the black rice protoplasts of *Cempo Ireng* Wild Type were very fragile so that it was impossible to centrifuge.

5. Transfection of pCK205 plasmids to protoplasts with PEG6000 solution

The transfection process was carried out after obtaining protoplasts from black rice *Cempo Ireng* Wild Type. Unfortunately, the resulting protoplasts in this research have not been successfully stored for a long time, the transfection process was carried out immediately after digesting the protoplasts on the same day. The incubation of the transfection process after adding the plasmid and PEG was carried out for ± 14 hours from 19.00-09.00 WIB. Based on the research of Zhang, the incubation process was carried out for 6-16 hours [10].



a b

Figure 3. Transfection results were observed with a confocal microscope. a) With PEG6000 30%. b) With PEG6000 40%

Observation with confocal microscopy showed that the result of transfection with PEG6000 concentration of 30% was more obvious than that of transfection with PEG6000 concentration of 40%. Most GFP signals are localized in the cytoplasm and plasma membranes [4].

Conclusions

Based on research results, using seedlings as main explants to produce protoplasts are easier to than callus and using PEG6000 30% for transfection was more effective than PEG6000 40%.

References

[1] Y. A. Purwestri, R. D. K. Sari, L. N. Anggraeni, and A. B. Sasongko, "Agrobacterium Tumefaciens Mediated Transformation of rolC:Hd3a-GFP in Black Rice (Oryza Sativa L. cv. Cempo Ireng) to Promote Early Flowering," *Procedia Chem.*, vol. 14, pp. 469–473, 2015, doi: 10.1016/j.proche.2015.03.063.

[2] J. M. Salgado, A. G. C. De Oliveira, D. N. Mansi, C. M. Donado-Pestana, C. R. Bastos, and F. K. Marcondes, "The Role of Black Rice (*Oryza sativa* L.) in the Control of

Hypercholesterolemia in Rats," *J. Med. Food*, vol. 13, no. 6, pp. 1355–1362, Dec. 2010, doi: 10.1089/jmf.2009.0246.

[3] F. A. Susanto, P. Wijayanti, A. N. Fauzia, R. D. Komalasari, T. R. Nuringtyas, and Y. A. Purwestri, "Establishment of a plant tissue culture system and genetic transformation for agronomic improvement of Indonesian black rice (Oryza sativa L.)," *Plant Cell Tissue Organ Cult. PCTOC*, vol. 141, no. 3, pp. 605–617, Jun. 2020, doi: 10.1007/s11240-020-01819-0.

[4] F. He, S. Chen, Y. Ning, and G. Wang, "Rice (*Oryza sativa*) Protoplast Isolation and Its Application for Transient Expression Analysis," *Curr. Protoc. Plant Biol.*, vol. 1, no. 2, pp. 373–383, Mar. 2016, doi: 10.1002/cppb.20026.

[5] S. Toki *et al.*, "Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice," *Plant J.*, vol. 47, no. 6, pp. 969–976, Sep. 2006, doi: 10.1111/j.1365-313X.2006.02836.x.

[6] S.-L. Yao, Y. Xu, Y.-Y. Zhang, and Y.-H. Lu, "Black rice and anthocyanins induce inhibition of cholesterol absorption in vitro," *Food Funct.*, vol. 4, no. 11, p. 1602, 2013, doi: 10.1039/c3fo60196j.

[7] S. H. Wani, G. S. Sanghera, and S. S. Gosal, "An efficient and reproducible method for regeneration of whole plants from mature seeds of a high yielding Indica rice (Oryza sativa L.) variety PAU 201," *New Biotechnol.*, vol. 28, no. 4, pp. 418–422, Jul. 2011, doi: 10.1016/j.nbt.2011.02.006.

[8] P. Apridamayanti, R. Pratiwi, Y. A. Purwestri, W. A. Sri Tunjung, and R. Rumiyati, "Anthocyanin, nutrient contents, and antioxidant activity of black rice bran of Oryza sativa L. 'Cempo Ireng' from Sleman, Yogyakarta, Indonesia," *Indones. J. Biotechnol.*, vol. 22, no. 1, p. 49, Jan. 2018, doi: 10.22146/ijbiotech.26401.

[9] S. Kumar Sah and A. Kaur, "High Frequency Embryogenic Callus Induction and Whole Plant Regeneration in Japonica Rice Cv. Kitaake," *Rice Res. Open Access*, vol. 2, no. 2, 2014, doi: 10.4172/jrr.1000125.

[10] Y. Zhang *et al.*, "A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes," *Plant Methods*, vol. 7, no. 1, p. 30, Dec. 2011, doi: 10.1186/1746-4811-7-30.

[11] E. Chamani, S. K. Tahami, N. Zare, R. Asghari-ZAKARIA, M. Mohebodini, and D. Joyce, "Effect of Different Cellulase and Pectinase Enzyme Treatments on Protoplast Isolation and Viability in Lilium ledebeourii Bioss.," *Not. Bot. Horti Agrobot. Cluj-Napoca*, vol. 40, no. 2, p. 123, Nov. 2012, doi: 10.15835/nbha4028055.

[12] Kim, Jong-Kun, Kim, Jin-Hee, Kong, Won-Sik, and Kang, Hee-Wan, "Selection of High Efficient Enzyme for Protoplasts Isolation from Mushrooms," *Korean J. Mycol.*, vol. 38, no. 1, pp. 21–24, Jun. 2010, doi: 10.4489/KJM.2010.38.1.021.

[13] R. Ren *et al.*, "Highly Efficient Protoplast Isolation and Transient Expression System for Functional Characterization of Flowering Related Genes in Cymbidium Orchids," *Int. J. Mol. Sci.*, vol. 21, no. 7, p. 2264, Mar. 2020, doi: 10.3390/ijms21072264.