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PROTOPLASTS FUSION OF PHALAENOPSIS AND DENDROBIUM *)

Fusi protoplas *phalaenopsis* dan *Dendrodium*

Issirep Sumardi, Ari Indrianto and Endang Semiarti **)

ABSTRACT

Protoplasts of *Phalaenopsis* and *Dendrobium* were isolated enzymatically. The protoplasts of *Phalaenopsis* are easily distinguished from the protoplasts of *Dendrobium* because they were purple in colour, and after enzymatic digestion the chloroplast were in cluster at a certain site of the protoplast.

The fusion of both protoplast were done using various concentration of Polyethylene Glycol (PEG) and osmoliticum stabilizer. The first fusion occured 5 minutes after both protoplasts were mixed in 35 % of PEG with 0,3 M glucose and 50 mM $CaCl_2$ while other than 35 % were not effective.

keywords : protoplasts isolation - protoplasts fusion

ABSTRAK

Isolasi protoplas *Phalaenopsis* dan *Dendrobium* dengan menggunakan enzim, mengasilkan isolat dengan ciri berbeda. Protoplas Phalaenopsis berwarna ungu dan kloroplasnya mengumpul pada suatu tempat tertentu dalam ptotoplas.

Kedua macam protoplas difusikan menggunakan PEG dengan beberapa konsentrasi dan asmolitikum. Fusi dapat terjadi kurang dari 20 menit dalam larutan 35 % PEG, 0,3 M glukose dan 50 mM $CaCl_2$.

kata kunci : isolasi protoplas - fusi protoplas

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**) Faculty of Biology, Gadjah Mada University, Yogyakarta.

INTRODUCTION

Since 1960 Cocking had developed techniques to isolate protoplasts by enzymatic digestion of the cell wall. Plant protoplasts play important role in the genetic manipulation.

Power *et al.* (1970) and Cocking (1971) fused somatic cells for the first time using protoplast of maise and oat, despite the fact that they did not obtain hybrid plants as a result of the fusion. In 1971 Takebe *et al.*, and Takebe and Nagata (Piereik, 1978) obtained normal plant from tobacco protoplast.

Fusion of protoplasts is generally induced by incubation in high concentration of Polyethylene Glycol (PEG), Ca^{++} and a relatively high pH (Menczel, 1983 *cit.* Pierik, 1978). The PEG solution and Ca^{++} ions were washed away after fusion has taken place.

Taniguchi *et al.* (1989) has studied the fusion process of rice and lettuce using electric field for inducing fusion. In this paper the author observed the indentifying mark of protoplasts of *Dendrobium* and *Phalaenopsis*, and the process of protoplast fusion of both orchids microscopically.

MATERIALS AND METHODS

1. Protoplast Isolation
   Protoplasts were isolated directly from leaves of both orchid. The leaves of seedlings (sterile condition, from seed culture) were cut into small pieces, and then treated with enzyme solution (Table 1), without shaking, kept in the dark at 25°. After incubation time, isolated protoplast were filtrated with nylon filter (80 μ m), and washed with washing medium by centrifugation for 3 minutes each at 100 g. The precipitates were purified with purification medium by centrifugation. Composition of washing medium and purification medium were presented in Table 2 and Table 3.

Table 1. Composition of Washing Medium (in 50 ml sol.)

	! EM_1	! EM_2
Cellulase Onozuka R - 10	! 500 mg	! 500 mg
Macerozyme R - 10	! 50 mg	! -
Pectolyase Y - 23	! -	! 50 mg
Mannitol	! 4.500 mg	! 4.500 mg
pH	! 5.6	! 5.6

Table 2. Composition of Washing Medium (in 50 ml sol.)

Mannitol	! 4.550 mg
$CaCl_2$. 2 H_2O	! 1.740 mg
MES *)	! 500 mg
pH	! 5.6

*) MES = Morpholino Ethano Sulfoxyde

Table 3. Composition of Purification Medium (in 50 ml sol.)

Sucrose	! 10.720 mg
MES	! 500 mg
pH	! 5.6

2. *Polyetylene Glycol (PEG) solution*
   PEG (WM 6000) solutions were used as inducing agent for protoplast fusion (Constabel, 1984). The solution consists of various osmotic stabilizer, mixed with various concentrationof PEG (see Table 4).

Table 4. Composition of inducing agent solution

	P_1	P_2	P_3	P_4	P_5	P_6	P_7
Glucose	0,3 M	0,3 M	0,3 M	0,3 M	0,3	-	-
$CaCl_2.2H_2O$	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
PEG 6000	20 %	25 %	30 %	35 %	40 %	30 %	30 %
Mannitol	-	-	-	-	-	9 %	-
Sucrose	-	-	-	-	-	-	0,6 M
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8

3. *Protoplast Fusion*
In this experiment microdroplet method were used for protoplast fusion. Small drops of viabel protoplast from both orchid were dropped on the glass added with aliquot fusogent. The process of the fusion were observed microscopically.

RESULTS AND DISCUSSION

1. *Protoplast Isolation and Marker*
After 3 -4 hours incubation more than 75 % of protoplasts were isolated completely. The effective time for wall degradation were 3 hours for EM_2, and more than 4 hours for EM_1. The number of protoplasts isolated in EM_2 were 85 %, while in EM_1 were 80 %.

The protoplast of *Phalaenopsis* were easily distinguished from *Dendrobium* protoplast, because the former had a purple colour, the size were smaller than *Dendrobium* protoplast, and the chloroplast were in cluster at a certain site of the protoplast. *Dendrobium* protoplast were bright with green chloroplast in the centre, their size were bigger.

2. *Viability of protoplast*
After purification the protoplasts were floating at the surface of the purification medium, and than their viability were tested. Using Evan Blue stain, the viabel protoplast blocked the stain in the outside of the membrane, while the broken protoplasts were stained completely.

According to Kanai and Edwards (1973) the broken protoplast were easily perforated by stain. The density of protoplasts were also counted. Marker indentification and viability test of each protoplast were necessary because after somatic hybridization there were the biggest problem to out select the results of the fusion, whether hybridization carried out by the same protoplasts or from different protoplasts (Pierik, 1987).

3. *Protoplast Fusion*

The viable protoplast of both orchid then mixed, and various concentration of inducing agents were added. There were different percentage of fusion on each treatment. Fusion with p_4 (Table 4) showed higgest persentage, i.e. 83.44%, while P_1, P_2, P_3, P_5 about 60 %, and P_6, P_7, less than 40 %.The succes of protoplasts isolation and fusion depend on the tissue donor condition (Contabel, 1970), and in this case also PEG concentration. The concentration of PEG for inducing agent and in this case PEG have generally 25 -40 %. (Constabel, 1984). In some plants 40 % of PEG caused agglutination between protoplasts, so the solution needed high concentration of Ca^{++} addition. pH value and concentration of Ca^{++} affected the yield of protoplasts fusion. The first fusion between protoplasts appeared 5 minutes after treatment, by the contact of two membrans. The complete fusion occured about 16 - 18 minutes after treatment. There were two conditions of fusion. The first, fusion between the same protoplasts (self fusion, spontaneous fusion) produced homokaryon. The second, fusion between two different protoplasts (somatic hybridization), *Dendrobium* protoplast x *Phalaenopsis* protoplast, produced heterokaryon. We also noted that there were found more than two protoplasts fused, and made cluster of protoplasts (multi fusion). Actually, the higgest percentage of fusion produced by P_5. Glucose is the best osmoloticum stabilizer compared with mannitol and sucrose. PEG solution mixed with mannitol or sucrose were not effective, produced lower percentage of fusion. In P_1, P_2, P_3 and P_4 different concentration of PEG glucose played significant role on fusion process. After both protoplasts fused, they would be washed by sorbitol or mannitol solution about 10 - 15 minutes. In this case PEG affected the stability of membrane protoplasts.

Somatic hybridization can used as an alternative method to create hybrid which would not be possible by normal crossing.

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