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The optimization mesophyll protoplast isolation for *Phalaenopsis amboinensis*

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Abstract

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Indonesia has 25 species of month orchids and 10 of them are endemic to Indonesia. Plant Conservation Center (LIPI) conserved 15 species of orchids which means that it has been conserving as much as 23.44% of the orchids in the world (Rahayu, 2015). Orchid conservation efforts in *Phalaenopsis amboinensis*, both *ex vitro* and *in vitro* continue to be made in order to increase biodiversity. One of them is made by protoplast fusion approach that begins with the isolation of protoplast *Phalaenopsis amboinensis.* This study aimed to produce viable protoplast of *Phalaenopsis amboinensis* by developing procedure for mesophyll protoplast isolation. Protoplast is isolated from young orchid leaf. This experiment was conducted on six combinations of enzymes (Cellulase concentrations of 1%, 1.5%, 2% and macerozyme 1%, 1.5%) and two incubation in the dark (4 h and 6 h), so the total combinations become 12. The method of analysis used a completely randomized design. The viable protoplast result was evaluated. The result showed that the best achieved enzyme combination was Cellulase 2.0% + Macerozyme 1.0% and incubation time of 6 h; compared to other treatments of forming spherical protoplasts and chloroplasts it tends to clump together and solid. The number of protoplasts 1.1×10^6 and the percentage of viable protoplasts 83.88 %.

Keywords: protoplast; isolation; *Phalaenopsis amboinensis*

Introduction

There are more than 60 species and several natural hybrid *Phalaenosis* spread throughout Asia with tropical climate to Pacific and Australia. The orchid collections found in the Bantimurung-Bulusaraung National Park are very diverse. *H. beccarii* is endemic to Sulawesi and the Moluccas. Similarly, *Phalaenopsis amboinensis* is found growing only in eastern Indonesia (Puspitaningtyas, 2017).

Phalaenosis is a species of orchid that is most common being hybridised whose hybrids are most listed in world market *Phalaenopsis* is well known for its beauty and has been popular as a *genus* of orchid functioned as ornamental plants as a result of beautiful cultivar production from interspecific as well as inter-genetic hybridization (Shrestha, et al,. 2007), freely around Gowa to Poso This flower is widely traded in the district of Sulawesi island, and even more throughout Indonesia. *Phalaenopsis amboinensis* is one of the vulnerable orchids which is protected by Government Regulation No.7/1999, the month Sulawesi orchid (*Phalaenopsis amboinensis*) is one of the endemic orchid species protected now by the government as endangered. Besides the government, breeders and botanists also can contribute to preservation of the endemic plants. Germplasm conservation efforts of orchids can be carried out in his native habitat in situ and outside their natural habitats ex situ (Rosdiana, 2010).

That prompted orchid uncontrolled exploitation gave effect on its existence and preservation (Rosdiana, 2010). The attempt to produce commercial hybrid orchid is done interspecifically with conventional hybridization technique

due to great difficulty in doing so by inter-genetic technique. The current somatic hybridization protocol, which is applied to wheat plant, has succeeded to produce symmetric and asymmetric somatic hybrids and its derivatives (Liu & Xia, 2014).

Other application of biotechnological tools is regeneration from protoplasts culture. This system allows applying protoplast fusion technology for facilitating gene transfer between incompatible rose species holds great potential. Application of this technique allows one to bypass sexual incompatibilities thus facilitating widening of the gene pool available for rose improvement (Ginova et al., 2012). Protoplast fusion method development using protoplast isolation is initialized by protoplast isolation as hybrid plant material resulted from fusion; this is called Somatic hybridization. Protoplast fusion does not require any sexual reproduction process and it enables the combination of the desired parental generation despite the taxonomic relation (Bhojwani & Prem, 2013).

Protoplast isolation is common for various species the protoplast itself has totipotent potential. Therefore, when protoplast is given proper chemicals it is able to form new cell walls. Further, in 20th century the genetic modification is developed through protoplast fusion and transformation (Davey et al., 2005).

Plant cell is usually protected by rigid cell consisted of cellulose which supports the plants structures. Cell walls can be degraded by enzyme that contains cellulose and macerozyme in order to produce protoplast (Wu et al., 2009). Protoplast isolation can be done by enzymatic process. Enzymatic method is a general method in protoplast isolation for its ability in producing a great number of viable protoplast. Each plant has different respond toward enzyme composition and concentration. The combination of cellulase R-10 and Macerozyme R-10 enzymes have been commonly used. However, differences in cell wall composition from different source materials, require optimization of protoplast isolation (Huo et al., 2017). The results showed that one of the successes of protoplast isolation was determined by the composition and concentration of the enzyme, as reported by Pindel (2007) on *Cymbidium* sp. L., using Cellulase 3.2%, Macerozyme 1.2% and Peptinase 0.5%, while Kanchanapoom et al. (2001) using cellulase enzyme 1% and 1% macerozyme on *Dendrobium panpodour* orchids. While on the plant *Etlingera elatior* the best combination is 3% Cellulase Onozuca "R-10, 2% Meicelase and 1% Driselase. Combination of 0.5% Cellulace enzyme and 0.1% Macerozym can be used to isolate protoplasts of orophid orchids *paraphalaenopsis laycockii* (Wida Utami & Hariyanto, 2015).

The incubation time at the time of protoplast isolation will affect the amount of protoplast viabel. Based on the results of research conducted by Khentry et al. (2006), that on the *Dendrobium* Sonia Orchid "Bomb 17" requires an incubation time of 5 hours, although not significantly different from 3, 4, 6 hours. Furthermore, the time required by the *Dendrobium crumenatum* orchid is 4 hours and if more than 4 hours the amount of protoplasts produced decreases (Tee et al*.,* 2010). Kanchanapoom et al. (2001) reported using a 3-hour incubation period for the isolation of *Dendrobium pompodour* orchid protoplasts. The mentioned above report shows that every species of plants, especially orchids required a combination of concentration of enzymes, osmotikum and different incubation time.

The objective of this study was to develop a procedure for isolation of protoplasts of the physienotic orbit leaf of *Phalaenopsis amboinensis* to obtain the viable protoplast by treatment of combination of enzyme concentration and different incubation times.

Materials and Methods

The research was conducted at the Laboratory of Plant Culture Network, Department of Biology, Faculty of Science and Technology, Airlangga University, Surabaya and Biotechnology Laboratory University of Muhammadiyah Malang. The study was conducted for 3 months.

Plants used as a source of protoplasts are leaves of orchids *Phalaenopsis amboinensis* in bottles (*in vitro*) aged 8-10 months (Fig. 1a,b) were obtained from the breeding laboratory and orchid development Handoyo Budi Orchids, Malang-East Java.

Protoplast isolation

Leaf explants weighed 1 g/treatment, the upper and lower epidermis slashed (Fig. 1c). Then the mesophyll was cut across 1 mm wide (Fig. 1d). Insert the mesophyll piece into a 6 cm petri dish containing 5 mL of the enzyme solution according to the tested treat (Fig. 1e), incubating in dark space over 4 and 6 hours in dark conditions. Combination of E1 enzyme treatment (1.0% Cellulase Enzyme + Macerozyme 1.0%); E2 (Cellulase Enzyme 1.0% + Macerozyme 1.5%); E3 (Cellulase Enzyme 1.5% + Macerozyme 1.0%); E4 (Cellulase Enzyme 1.5% + Macerozyme 1.5%); E5 (Cellulase Enzyme 2.0% + Macerozyme 1.0%); E6 (Cellulase Enzyme 2.0% + Macerozyme 1.5%). The enzyme used Cellulase Onozuka R-10 (PlantMedia Ltd Lot # V16110700) and Macerozyme Onozuka R-10 (Plant Media Ltd Lot # V16110700). All enzyme solutions are added 0.5 M sorbitol osmotikum (Tee, 2010) and 5 mM MES, pH 5.8).

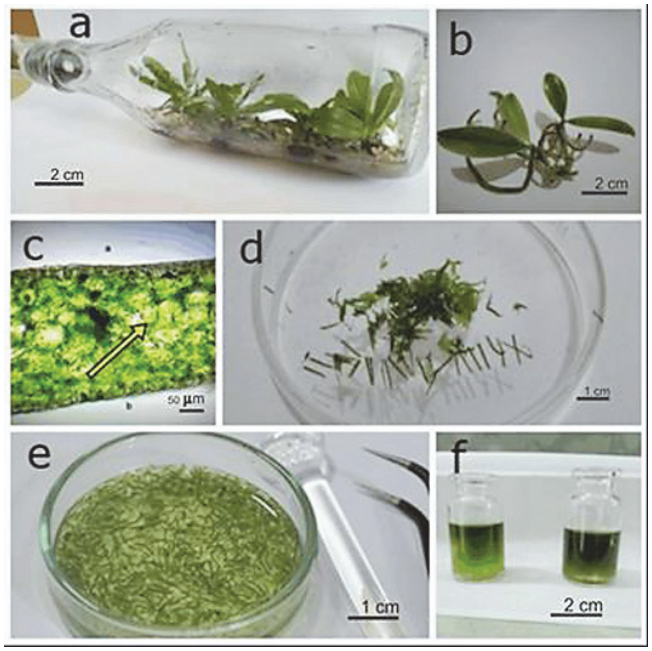

Fig. 1. Protoplast isolation process *Phalaenopsis amboinensis in vitro* (*a, b*); mesophyll (*c*); transverse slices of leaf 1 mm (*d*); enzyme solution (*e*); supernatant (*f*)

The result of protoplast isolation is filtered with nylon filter to remove the uncut tissue and debris. The protoplast suspension is inserted into vials of pre-treated wash solution of 5 mL Sucrose 0.5 M and 5 mM $CaCl_2$ 2 H_2O using a micropipette slowly (Fig. 1f). Then the protoplasts will float on the surface. The floating prototype taken 100 µL is inserted into an ependorf already containing 900 µL of sucrose solution of 0.5 M. The viable protoplast can be seen by adding 0.1 mL of Fluorescein diacetate stock solution (0.5 mg FDA dissolved with 1 mL acetone) and mixed 10 mL purifying solution. Then the protoplasts are seen using a UV microscope (Babaoúlu, 2000).

Observation of protoplast isolation results

The protoplasts were calculated using a haemocytometer under a microscope with magnification of 10 × 10 (100×) protoplast taken 25 µL was inserted into the camber haemocytometer and covered the glass cover. The total protoplast density is calculated in the camber box consisting of both viable and non viable protoplasts. The protoplast calculation uses the equation (Bastidas, 2016):

$$S = \frac{X}{L \times t \times P} \times 10^3.$$

Information

S = Protoplasts density

X = The average number of protoplasts in the chamber was observed

L = Counted area of 25 chambers = 0.1 mm

T = Chamber depth (0.1 mm)

P = Dilution

10^3 = Conversion constant from mm^3 to mL

The number of protoplasts by counting the total protoplasts consisting of non-viable protoplasts and viabel protublasts. The non viabel protoplas is shaped irregular (broken), while the protoblast viabel is round. The number of protoplast percentages viabel way:

$$\frac{\sum \text{Protoblast viabel}}{\sum \text{total protoplast}} \times 100\% =$$

Analysis design

The design of this study used a Factorial Completely Randomized Design. Factor 1 was the combination of enzyme concentration E1, E2, E3, E4, E5, E6, while factor 2 was T1 incubation time (4 hours) and T2 (6 hours). The results of protoplast isolation were analyzed using ANOVA and tested further using *Duncan's Multiple Range Test* 5% (DMRT).

Result

The isolation of protoplasts using the mesophyll tissue of the orchid *Phalaenopsis amboinensis* to accelerate cell wall degradations done in *Populus* plant with mesophyll tissue by modifying protoplast isolation procedure to adjust the enzyme concentration and isolation time to obtain 1×10^7 in 1 g of leaf (Guo et al., 2012). The wax coating on the *Liriodendron* hybrid plant can inhibit protoplast isolation, differences in cell wall composition from different source materials require optimization of each isolation (Huo et al., 2017).

The statistical analysis of protoplast density from isolation showed that E4 (1.5% Cellulase Enzyme + Macerozyme) yielded the highest density 9.6×10^5 compared with other treatment, although not significantly different with E5 treatment (Enzyme Cellulase 2.0% + Macerozyme 1.0%) 9.5×10^5 and E2 (Cellulase Enzyme 1.0% + Macerozyme 1.5%) 9×10^5 (Fig. 2A). According to Huddy et al (2013), work synergies of some enzymes necessary to degrade cell walls and protoplast discharges may depend on the nature of cellular complexity.

The result of percentage of viable protoplast showed (Fig. 2B) that treatment of E2 (1.0% Cellulase Enzyme + Macerozyme) produced 85.46% via protoplast signifi-

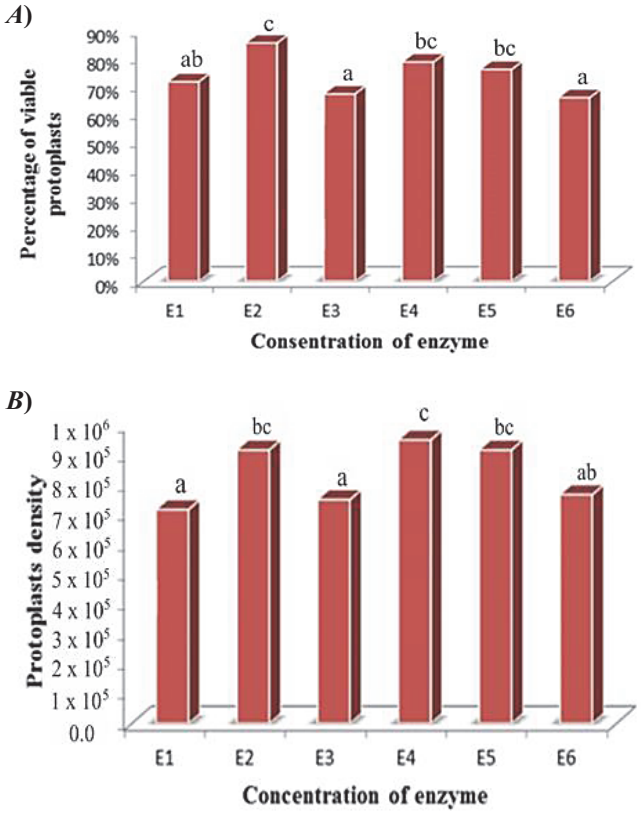

Fig. 2. Effect of enzyme concentration on (*A*) Mean protoplast density of isolation and (*B*) percentage of viabel protoplast from mesophyll of *Phalaenopsis amboinensis* orchid leaf (105/g wet weight). Enzyme E1 (1.0% Cellulase Enzyme + Macerozyme 1.0%); E2 (Cellulase Enzyme 1.0% + Macerozyme 1.5%); E3 (Cellulase Enzyme 1.5% + Macerozyme 1.0%); E4 (Cellulase Enzyme 1.5% + Macerozyme 1.5%); E5 (Cellulase Enzyme 2.0% + Macerozyme 1.0%); E6 (Cellulase Enzyme 2.0% + Macerozyme 1.5%). The numbers accompanied by the same letter are not significantly different in the DMRT test (0.05)

cantly different from other treatments, except with treatment E4 (1.5% Cellulase Enzyme + Macerozyme 1.5%) resulting in a 78.61% viable protoplast and E5 (Cellulase Enzyme 2.0% + Macerozyme 1.0%) with 75.97% of viable protoplasts (Figure 2B). The concentration of enzymes greatly influenced the yield and viability of protoplasts by using Pectolyase Y-23 and 0.1% (w / v) Pectolyase Y-23 was optimum, while the higher Y-23 Pectolyase concentration (0.25% w / V) does not increase the number of *Liriodendron* hybrid viable protoplasts (Huo et al., 2017). In addition, osmotic stabilizers play an important role for protoplast isolation with the addition of NaCl to help increase the maximum isolation of protoplast yield (Wubie et al., 2014). The use of MES buffers has an important role in maintaining low pH in protoplast isolation (Guy & Reinhold., 1978).

In this study, the protoplast density and percentage of different viable protoplasts were obtained. Effect of incubation time, the result of protoplast density at 6 hours resulted in 1.01 X 10^6 (Fig. 3A) and the percentage of viable protoplast reached 79.95% (Fig. 3B). Isolation of protoplasts on plant *Dalbergia sissoo* Roxb concentration of enzyme solution only 0.5% pectinase and 1.5% cellulose used to degrade middle lamella mesophyll tissue required incubation for 6 hour (Mukhtar et al., 2012) while according to Tee et al. (2010) and on the isolation of *Dendrobium crumenatum* protoplast, *Dendrobium* "SoniaBom 17" takes 4 hours incubation time (Khentry et al., 2006). The proper incubation time for isolation of mesophyll protoplasts of orchids *Paraphalaenopsis laycockii* is 4 hours (Utami & Hariyanto, 2015). The incubation time of protoplast isolation in *Dendrobium crumenatum* longer than 6 hours may cause protoplasts to rupture (Tee et al., 2010).

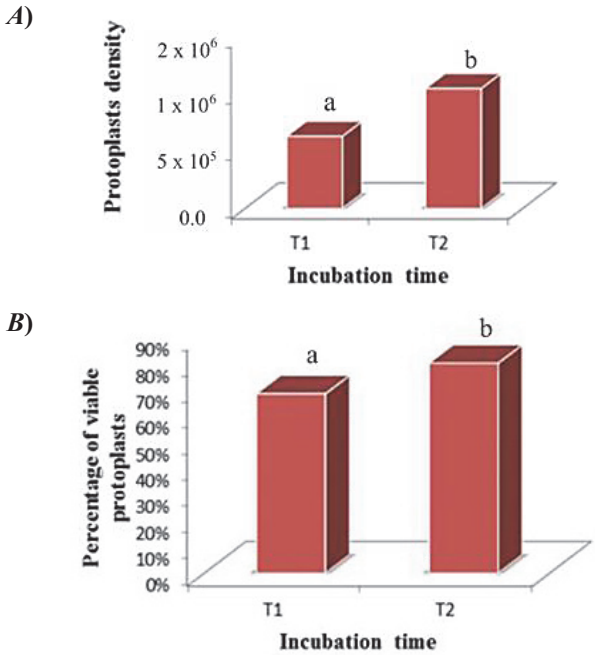

Fig. 3. Effect of incubation time on average. Protoplast density of isolation (*A*) and (*B*) percentage of viable protoplasts from mesophyll of *Phalaenopsis amboinesis* orchid leaf (105); T1 (4 hours); T2 (6 hours); the numbers accompanied by the same letter are not significantly different in the DMRT test (0.05)

The combination of enzyme concentration and incubation time, indicated the interaction of treatment to protoplast discharge in the presence of cell wall degradation in mesophyll tissue. This is based on observation of protoplast density and analysis of variance and continued with further test of DMRT (0.05). There was a significant interaction with E5T2 treatment (Cellulase 2.0% + Macerozyme 1.0%; 6 hours) yielding the highest density 1.1 X 10^6 compared to other treatments, except E1T2, E3T2, E4T2, E2T2 and E6T2. While the lowest protoplast density was found in E1T1 treatment (4.0 X10^5) (Fig. 4).

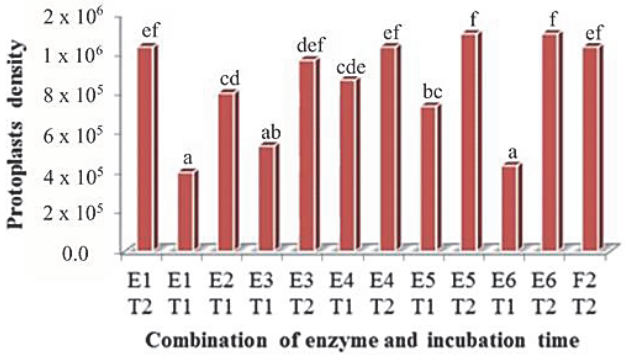

Fig. 4. Interaction of enzyme concentration and incubation time on average protoplast density result from mesophyll of *Phalaenopsis amboinensis* orchid leaf (1 g wet weight); Enzyme E1 (1.0% Cellulase Enzyme + Macerozyme 1.0%); E2 (Cellulase Enzyme 1.0% + Macerozyme 1.5%); E3 (Cellulase Enzyme 1.5% + Macerozyme 1.0%); E4 (Cellulase Enzyme 1.5% + Macerozyme 1.5%); E5 (Cellulase Enzyme 2.0% + Macerozyme 1.0%); E6 (Cellulase Enzyme 2.0% + Macerozyme 1.5%); T1 (4 hours) and T2 (6 hours)

According to Ratanasanobon and Seaton (2013) protoplast isolation in *Chamelaucium* group plants with 2% cellulase enzyme composition and 1% macerozyme and 6 hours more incubation time, protoplasts can't be released – different protoplast sources require different enzymes to isolate protoplasts because they have different intra and intercellular tissue compositions.

Interactions treatment occurs at the percentage of viable protoplasts on protoplast discharge. The result of variance analysis was very real and continued with further test of DMRT (0.05). There was a significant interaction with E2T2 treatment (Cellulase 1.0% + Macerozyme 1.5%; 6 hours) resulted in the highest protoplast percentage of 87.25% compared to other treatments except E5T2 (83.88%), E2T1 (83.66%), E1T2 (77.58%), E4T1

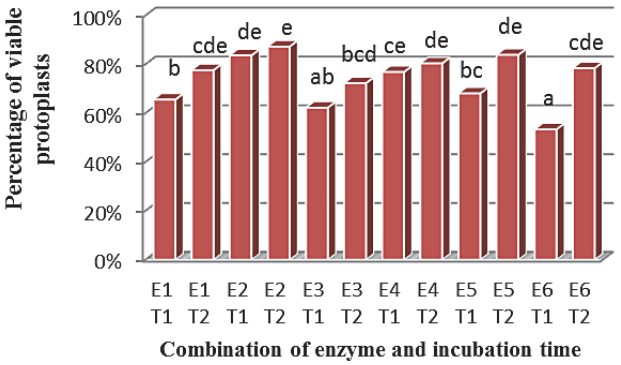

Fig. 5. Interaction of enzyme concentration and incubation time on percentage of viable protoplast from mesophyll of *Phalaenopsis amboinensis* orchid leaf (105/g wet weight); Enzyme E1 (1.0% Cellulase Enzyme + Macerozyme 1.0%); E2 (Cellulase Enzyme 1.0% + Macerozyme 1.5%); E3 (Cellulase Enzyme 1.5% + Macerozyme 1.0%); E4 (Cellulase Enzyme 1.5% + Macerozyme 1.5%); E5 (Cellulase Enzyme 2.0% + Macerozyme 1.0%); E6 (Cellulase Enzyme 2.0% + Macerozyme 1.5%); T1 (4 hours) and T2 (6 hours)

(76.85%), E4T2 (80.37%) and E6T2 (78.37%). While the lowest percentage of protoplast viable was found in treatment E6T1 (53.33%) (Fig. 5).

The viable protoplasm may be affected by hypotonic or hypertonic osmotic effect changes due to the sudden shifting force resulting from flow initiation or surface disconnection of two different solutions (Moshelion et al., 2004). The outbreak of protoplasts can also be caused by crystalline crystals with sharp structures such as needles can penetrate and break protoplasts (Fig. 6a). However, the use of sucrose, rapida crystals to be minimum due to the effects of flotation and precipitation (Kanchanapoom et al., 2001) and rapida crystals are present in the mesophyll, spon parenchyma (Kandemir et al., 2016). In the angiosperm of the general cranial form of intracellular and crystalline adjacent to a special vacuole cell crystals called idioblast, crystalline formations in idioblasts are usually associated with membranes, chambers found within the vacuola (Öztürk & Dane, 2014)

Non-viable protoplasts had a round shape and does not absorb the color when observed under a microscope fluorescein, while the viable protoplasts will issue a fluorescein green color of the FDA led to protoplasts become fluorescent and protoplast looked perfectly round (Fig. 6 b,c,d,e,f)

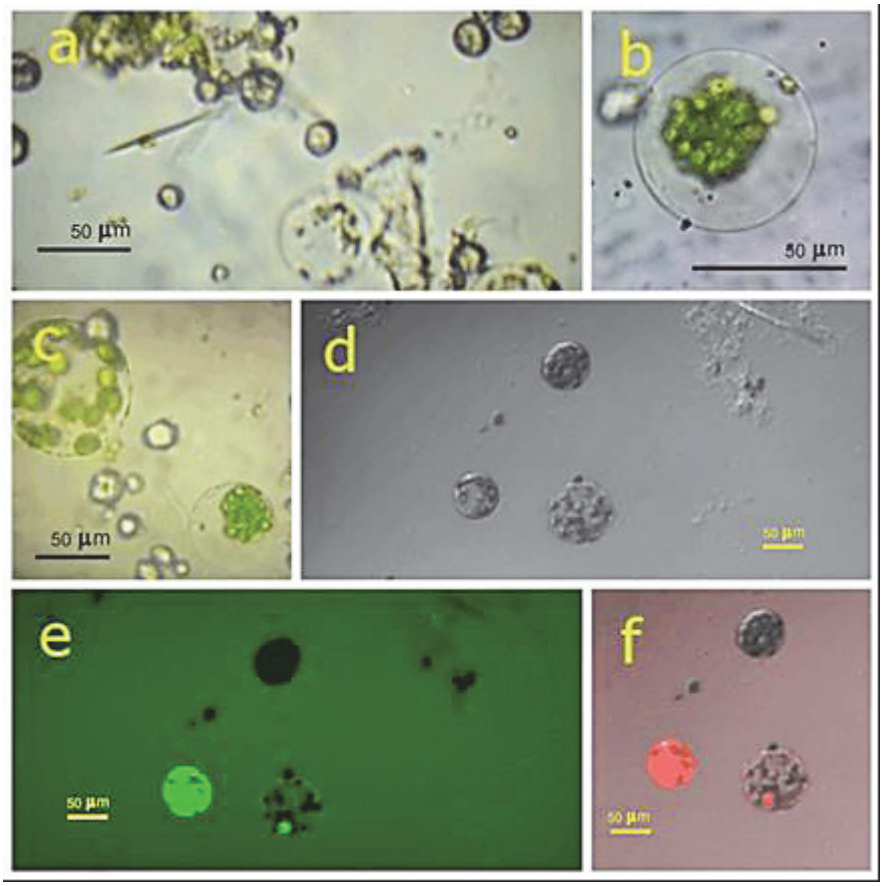

Fig. 6: Protoplast *Phalaenopsis amboinensis*;
Raphida (*a*); protoplast viable (*b, c*); FDA staining (*d, e, f*); non-viable (nv); viable (v)

Conclusion

The results of this study showed that the concentration of enzyme combination E5T2 (Enzyme Cellulase 2.0% + Macerozyme 1.0%) with 6 hours incubation time proved to influence protoplast density and viability significantly. The release of protoplasts is well-desirable. The insulation efficiency conditions of *Phalaenopsis amboinensis* leaf are self-derived *in vitro*. This study protocol can be used for advanced research studies in gene manipulation and protoplast fusion.

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