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Protoplast Fusion between Indonesian *Citrus maxima* (Burm.) Merr. and *Citrus reticulata* L.: A Preliminary Report

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ABSTRACT

Protoplast fusion is a useful technique for citrus genetic improvement. Establishment of protoplast technology could produce triploid seedless citrus and new citrus genetic combination. The aim of this research was to establish protoplast isolation, protoplast fusion between pummelo 'Nambangan' (*Citrus maxima*) and keprok 'Garut' (*C. reticulata*), and its protoplast culture. Protoplasts were isolated from leaves of *in vitro* seedlings of pummelo 'Nambangan' and embryogenic callus of keprok 'Garut' with two different compositions of enzymes. The results showed that optimum enzymes compositions for cells mesophyll wall degradation were consisted of 0.5 % Onozuka cellulase RS10, 0.5 % Macerozyme R10 and 0.1 % Pectolyase Y23. Optimum enzymes composition for callus embryogenic cells wall degradation were 0.5 % Onozuka cellulase RS10 added with 0.5 % Macerozyme R10. Protoplast fusion was conducted with PEG-mediated. Protoplast mixture were incubated in 40 % PEG for 8-10 min and after that could be observed 40.6 % of unfused protoplasts, 4.6 % of homofused mesophyll protoplasts, 28.2 % of homofused callus protoplasts, 7.8 % of heterofused and 9.6 % of multifused protoplasts from mesophyll and callus. Cells divided at the first week and began to form cell colonies afterwards in liquid culture.

INTRODUCTION

Protoplast fusion is an alternative technology to produce triploid plants, at once somatic cells combination of different cultivars, species or genus produce new genetic combinations. Protoplast fusion allows occurred material genetic combination from cytoplasm and nucleus of two parent cells (Abbate et al., 2012; Ananthakrishnan, Čalović, Serrano, & Grosser, 2006; Grosser & Gmitter, 1990). Type of protoplast incorporation in the fusion process that may occur is homofusion between leaf protoplasts or between callus protoplasts, heterofusion between leaf protoplast and callus protoplasts, multifusion among leaves and callus protoplasts (Hennig et al., 2015; Husni, 2010). Cytoplasm fusion accompanied with nucleus fusion would produce autotetraploid cell from homofusion and allotetraploid cells from heterofusion type (Grosser, Gmitter, Louzada, & Chandler, 1992).

Protoplast technology have been used for complement conventional breeding, one of which to provide parents tetraploid crosses with diploid parental to produce triploid plants which are seedless (Xie et al., 2015). Protoplast fusion can be used also for genes transfer in the development of new cultivars to overcome several problems including sexual incompatibility, embryo nucelar (polyembryoni), and male or female sterility (Abbate et al., 2012; Fu, Peng, Cai, & Guo, 2011; Grosser & Gmitter, 1990; Guo, Xiao, & Deng, 2013). Currently, the subcellular distribution of CsMYBF1 was analysed using transient expression in a citrus protoplast system (Liu et al., 2016).

Pummelo cv 'Nambangan' was one of commercialized Indonesian *Citrus maxima*. Indonesian Ministry of Agricultural issued a decree that Pummelo cv 'Nambangan' was a superior variety (Agricultural Ministerial decree No. 496/Kpts/TP.240/10/2000). Pummelo cv 'Nambangan'

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breeding programs aim to produce seedless fruit and improving fruit quality. Pummelo breeding program could be conducted by sexual hybridization (crossing) and somatic hybridization with mandarin type. Keprok Garut is one of potential traits of Indonesian *C. reticulata*. Keprok distinguished characteristic are juicy, thin-skinned, has a distinctive aroma, orange flesh color, fresh and sweet taste, fruit size ranges from 7-9 cm in diameter (decree of the Minister of Agriculture No.760/Kpts/TP.240/6/99). Pummelo 'Nambangan' have physical qualities and chemical qualities better than other cultivars during storage until 10 week after harvest (Fikrinda, Susanto, Efendi, & Melati, 2015).

Pummelo is monoembryogenic citrus, this character makes pummelo as an ideal female parent for sexual hybridization because eliminates the need for embryo rescue (Kainth & Grosser, 2010). Limitations in sexual hybridization of Pummelo and Mandarin type is not reported yet, but showed low achievement on Indonesian siam 'Pekanbaru' as male parent crossing with pummelo 'Nambangan' as female parent that was 7.1 % (Sukarmin & Ihsan, 2008). Therefore, protoplast fusion became an alternative solution to increase the possibilities of characters' combination from different citrus species through interspecies somatic hybridization.

Somatic hybridization between pummelo cv 'Nambangan' and keprok 'Garut' are expected to produce medium size diameter of pummelo fruit, the skin is thinner, the percentage of edible fruit portion is higher, resistant to CTV (*Citrus Tristeza Virus*) and a tetraploid plant. Protoplast fusion between pummelo and type of mandarin orange has been done to produce good rootstock attributes and an alternative rootstock restant to CTV (Ananthakrishnan, Čalović, Serrano, & Grosser, 2006), to develop seedless citrus (Xie *et al.*, 2015).

Protoplast fusion technology in citrus has not been established yet in Indonesia. Husni (2010) had combined Indonesian siam 'Si madu' with 'Satsuma' mandarin through somatic hybridization for transferring 'Satsuma' mandarin *Cytoplasmic Male Sterility* (CMS) gene to siam 'Si madu' for producing seedless fruit. Somatic hybridization between pummelo 'Nambangan' and keprok 'Garut' was conducted to establish Citrus protoplast fusion technology in Indonesia and supporting pummelo breeding programs. Keprok was chosen as a parent plant because of the ease peeling, good orange flesh and its ability to form somatic embryogenic

callus line. Pummelo has good quality in rooting systems and resistant to CTV. This research is expected to obtain the optimal composition of cell wall degradation enzyme, fusion method as well as protoplast culture. The objective of this research was to establish protoplast isolation, protoplast fusion between pummelo 'Nambangan' (*Citrus maxima*) and keprok 'Garut' (*C. reticulata*), and its protoplast culture.

MATERIALS AND METHODS

Protoplast fusion between Indonesian *Citrus maxima* (Burm.) Merr. and *Citrus reticulata* L. was detail observed in Plant Cell and Tissue Culture Laboratory, Research Center of Biotechnology, Indonesian Institute of Sciences during January-August 2015. Protoplast fusion technique that use in this study consists of enzymatic protoplast isolation, protoplast fusion induced by PEG and fused protoplast culture in liquid medium.

Protoplast Isolation

Protoplasts were isolated from leaves of *in vitro* Indonesian pummelo (*Citrus maxima*) 'Nambangan' germinated seeds and embryogenic callus of keprok 'Garut' (*C. reticulata*). Two kinds of enzyme solution compositions were used for tissue degradation. First composition was 0.5 % Onozuka RS Cellulase, 0.5 % Macerozyme R10 and 0.1 % Pectolyase Y23. Second composition was 0.5 % Onozuka RS Cellulase and 0.5 % Macerozyme R10. Component of enzyme solution was added with 0.7 M Mannitol, 24.5 mM CaCl₂, 0.92 mM NaH₂PO₄ and 6.15 mM MES as complements. The concentrations of enzyme stock solution were 2 % Onozuka RS Cellulase, 2 % Macerozyme R10 and 0.4 % Pectolyase Y23. Enzyme stock solution was diluted with BH3 medium in 1:3 ratio v/v (enzyme solution: BH3 medium) in a 12 ml total volume (Grosser & Gmitter, 1990).

Protoplast isolation was performed using one step method which developed by Grosser & Gmitter (1990). To isolate protoplasts, leaves of pummelo seedlings grown *in vitro* were cut into thin strips (1-2 mm) horizontally along the leaves midrib, and embryogenic callus clumps were chopped into small pieces to enhance enzyme penetration. Those tissues were incubated in a 12 ml total volume of enzyme solution diluted with BH3 medium using a 100 ml Erlenmeyer flask. Flasks containing tissue and enzyme solution was incubated for 16 h at 28 °C in the dark on a rotary shaker at 50 rpm.

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Protoplasts were first purified by passing the digestion mixture through a nylon sieve (50-100 μm mesh) to remove undigested cell clumps and debris. Filtrate precipitation was conducted by centrifugation at 800 rpm for 8 minutes (Hitachi Himac 6EL). After removing supernatants, the protoplast sediments and cell debris were re-suspended with 'bubbling' methods by adding *Cell and Protoplast Washing* (CPW) solution containing 5 ml of 25 % sucrose. The suspension was purified with gradient centrifugation with addition of 2 ml CPW containing 13 % Mannitol, added drop to drop on the top of sucrose layer (to avoid mixing), and suspension was centrifuged for 8 min at 800 rpm. Viable protoplasts usually form a band at the interface between the two layers. The protoplasts were removed carefully from the interface using a Pasteur pipet and washed in 5 ml BH3 medium at 800 rpm for 5 min centrifugation. Pellet containing protoplasts was re-suspended in an appropriate amount of BH3 medium. Purified protoplasts were then ready for further manipulations.

Protoplasts were observed under inverted microscope (Leica, DMIL LED) with 400 magnification, for counting protoplast diameter observed at 3 field of views and 10 protoplasts per field of views, for each treatment. Statistical analysis of protoplast diameter was done by Analysis of Variance (ANOVA).

Haemocytometer was used to count protoplast number and protoplast yield. Protoplast yield was calculated as average of 10 box with $1/16 \text{ mm}^2$ and 0.1 mm depth. Yield was defined as protoplast number per ml isolated from 1 g of keprok 'Garut' callus and from 500 mg of pummelo 'Nambangan' *in vitro* leaves.

Protoplast Fusion

Protoplast fusion was performed using PEG method (Grosser & Gmitter, 1990). Forty percent PEG (BM = 6000) solution, added with 0.3 M glucose, and 66 mM CaCl_2 at pH = 6. Elution solution A consisted of 0.4 M glucose, 66 mM CaCl_2 , 10 % DMSO at pH = 6, and solution B consisted of 0.3 M Glycin at pH 10.5. To avoid precipitation, solution A was mixed with B immediately before adding to fusion dishes. Solution A was mixed with B at 9:1 ratio (v/v).

Protoplast from embryogenic callus and leaves mesophyll was mixed with equal volume (1:1). Protoplast fusion was induced in sterile plastic

Petri dishes (60 x 15 mm). Into each fusion dish, 2 drops of protoplast mixture were added, and 2 drops of 40 % PEG were added immediately to the droplets. After 8-10 minutes, 2 drops of A+B solution were added into each fusion dish on the periphery of the protoplast mixture. After 12-15 minutes, 12 drops of 0.6 M BH3 medium were added. After 5 minutes all the fluid was removed gently from the dish with a Pasteur pipette and replaced it with 12-15 drops of 0.6 M BH3 medium. This washing procedure was repeated twice. Finally, 1.5-2.0 ml 0.6 M BH3 medium was added, and protoplast culture were placed into sealed 60 x 15 mm plastic Petri dishes, then incubated in the dark at 25-27 °C.

Type of protoplast fusion (homofusion, heterofusion and multifusion) was observed using inverted microscope (Leica DMIL LED) at 400 magnifications, after 8-10 minutes of PEG addition. Percentage of each fusion type was calculated at 10 field views from single Petri dish. The fused protoplasts were identified by the presence of chloroplasts from pummelo 'Nambangan' leaves and starch granules from embryogenic callus of keprok 'Garut'.

Fused Protoplast Culture

Medium for protoplast culture was liquid BH3 medium with 2 concentrations which were 0.6 M and 0.4 M (Grosser & Gmitter, 1990). Solid medium for the growth of cell colony was consisted of MW vitamin with addition of 3 mg L^{-1} BAP (Karyanti, 2013; Merigo, 2011).

Fused protoplasts and protoplasts from embryogenic callus of keprok 'Garut' were cultured at Petri dish (60 x 15 mm) in 8-12 drops of 0.6 M BH3 medium. The same medium was added every week to maintain humidity. Protoplast culture was incubated in the dark at 25-27 °C for 4-6 weeks.

Protoplast growth phase was observed using inverted microscope (Leica DMIL LED). Cell colony was formed during fourth weeks; micro callus formation was induced with decreasing medium osmolarity by adding with 10-12 drops of 0.4 M BH3.

Early growth parameters were counting by the amount of cell colony and cell colony diameter formed at 10 Petri dish at fourth week using stereo microscope (Leica EZ4HD) with 8 magnifications. Each petri represented by single field view with 142.45 mm^2 wide. T test was conducted for data analysis of each parameter.

Callus which reached 0.5-1.0 mm in diameter

was transferred to solid medium consisted of MW basal medium containing 3 mg L⁻¹ BAP for callus embryogenic regeneration. Cell colony in solid medium was observed by microscope stereo (Leica EZ4HD) with 35 magnification.

RESULTS AND DISCUSSION

Protoplast Isolation

Two compositions of the enzymes which used in this study successfully released cell from leaves and callus tissues then degrading cell wall to release the protoplasts in the incubation process for 16 hours. Gradient centrifugation process on the protoplast isolation resulted in protoplasts layers at the interphase. Green layer was protoplast pellet from leaves mesophyll of pummelo ‘Nambangan’ and creamy layer was protoplast pellet from keprok ‘Garut’ callus. Pellet protoplast at interphase having good quality of protoplasts (intact and spherical), while the cell debris was precipitated on the bottom of the tube. These results indicated that the centrifuge method with mannitol and sucrose which have different molecular weight effectively separated protoplasts from cell debris. Methods of

separation by gradient centrifugation was effectively applied also to purified other pummelo protoplasts (Ananthakrishnan, Čalović, Serrano, & Grosser, 2006; Grosser, Gmitter, Louzada, & Chandler, 1992).

Characteristic of protoplasts from pummelo leaves and keprok callus are presented in Fig. 1. Protoplasts color of mesophyll cell was green because the cytoplasm contained chloroplasts, whereas protoplasts from callus of keprok ‘Garut’ was white/cream because the cytoplasm contained starch grains and vacuoles. Different characteristic of mesophyll and callus protoplast will facilitate microscopic identification of heterokaryons following fusion because its cytoplasm will contain chloroplasts and starch grains (Grosser & Gmitter, 1990).

Protoplast had spherical shape with average diameters were significantly different between mesophyll protoplasts and callus protoplasts but protoplast diameter was in a wide range (Table 1). This data shows that protoplasts size was vary and not determined by the composition of enzyme.

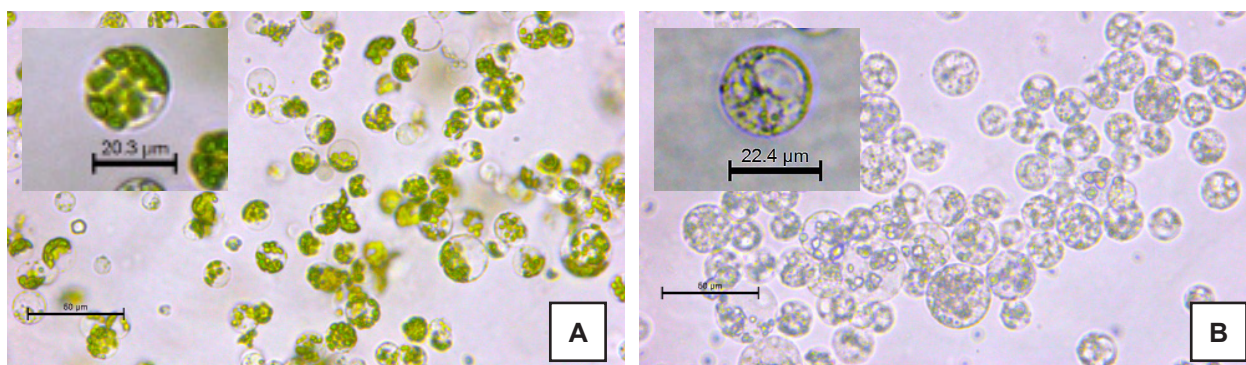


Fig. 1. Protoplasts from enzymatic isolation observed with microscope with 40 magnifications, (A) cell mesophyll protoplast from pummelo ‘Nambangan’ leaves, (B) embryogenic cell callus protoplast of keprok ‘Garut’ (bar = 50 µm)

Table 1. Protoplast diameter and yield from enzymatic degradation of leaves pummelo tissue and keprok callus

Explants	Cellulase RS (%)	Macerozime R10 (%)	Pectoyase Y23 (%)	Mean of protoplast diameter (µm)	Range of protoplast diameter (µm)	Protoplast density (protoplast mL ⁻¹)
Pummelo leaves	0.5	0.5	0.1	21.00a	13.99-27.07	5.02 x 10 ⁶
Pummelo leaves	0.5	0.5	0	18.24ab	11.85-33.77	3.31 x 10 ⁶
Keprocallus	0.5	0.5	0.1	16.68b	9.95-31.56	1.10 x 10 ⁶
Keprocallus	0.5	0.5	0	16.96b	12.56-20.95	2.08 x 10 ⁶

Remarks: For each column, mean followed by different letters denote statistical differences among treatments within each experiment according to ANOVA (P = 0.05)

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Table 1 also shows that cell wall degradation enzyme only affect on protoplast yields. The enzyme compositions containing 0.1 % Pectolyase Y23 produced higher yield from pummelo leaves, but produced lower yield from keprok callus. This shows that Pectolyase Y23 could be omitted for keprok callus cell wall degradation. Minimum yield required for protoplast fusion and its cultured was 1×10^5 protoplasts mL^{-1} (Vardi & Galun, 1989) was obtained from both enzyme compositions. Protoplasts produced from keprok callus and pummelo leaves in this experiment reached a sufficient amount, which ranged from 1.10×10^6 to 5.02×10^6 . Cellulose enzyme serves to release cell from plant tissues, whereas Macerozym and Pectolyase serves to degrade cell wall than resulting spherical protoplasts.

Protoplast Fusion

The addition of 40 % PEG solution and incubation for 8-10 minutes caused protoplasts plasma membrane attraction, eventually forming a connecting bridge and merging the cytoplasm (agglutination and aggregation process). Protoplast agglutinated to one another at random and causes the formation of several types of fusion. Agglutination between protoplasts with PEG assistance occurred because PEG molecules are capable of forming hydrogen bonds with water, protein, carbohydrates and other compounds with positively charged including Ca^{2+} from $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ added in the solution (Bhojwani & Razdan, 1983).

Different type of fusion was observed in this study (Fig. 2), meanwhile the average number of events is in Table 2. In this experiment, homofusion between protoplast from leaves infrequently happened, homofusion between keprok callus protoplasts was more frequent, 40.69 % of protoplasts were unfused. This was due to the density of protoplasts from pummelo leaves that was higher than keprok callus protoplasts. According to Grosser & Gmitter (1990), a high protoplast density will reduce the frequency of fusion occurrence. It is, therefore, necessary to dilute protoplast density by adding medium BH3 0.6 M so that PEG was able to penetrate between protoplasts and increased the protoplast membrane attachment.

Homofusion may produce autotetraploid regenerants and heterofusion may produce allotetraploid regenerants. Autotetraploid regenerants obtained an extra chromosome from the same source of protoplast while allotetraploid regenerants obtained an extra chromosome from the different source of protoplasts (Grosser, Gmitter, Louzada, & Chandler,

1992). In this study, culturing homofusion protoplast from pummelo or keprok would produce autotetraploid pummelo or autotetraploid keprok plants. Autotetraploid citrus are useful for breeding parent in interploidy crosses with diploid plant and resulting triploid citrus. Culturing heterofusion and multifusion protoplast will produce hybrid plant between pummelo and keprok. Hybrid plant between pummelo and keprok could be candidate for good quality rootstock (Ananthakrishnan, Čalović, Serrano, & Grosser, 2006) or candidate for scion improvement (Fu, Peng, Cai, & Guo, 2011).

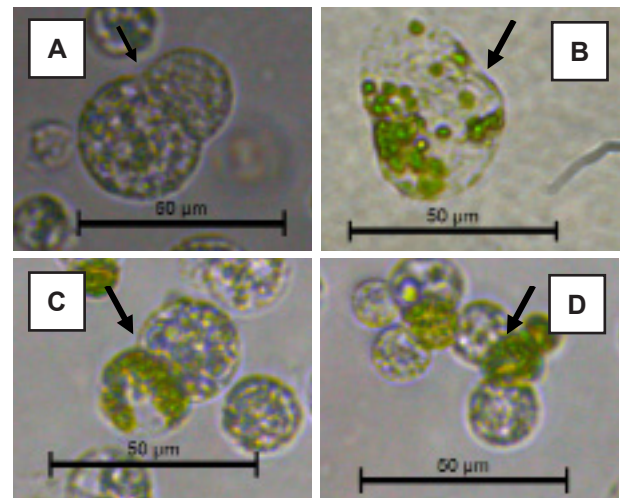


Fig. 2. Type of protoplast fusion after 8-10 minutes incubation in 40 % PEG: (A) Homofusion between protoplasts of callus keprok 'Garut', (B) Homofusion between protoplasts of pummelo 'Nambangan' leaves, (C) Heterofusion between protoplasts of pummelo 'Nambangan' leaves and callus keprok 'Garut', (D) Multifusion between several protoplasts from pummelo 'Nambangan' leaves and callus keprok 'Garut'. Inverted microscopic observation was conducted with 40 times magnification (bar = 50 μm)

Table 2. Percentage each type of fusion on incubation time 8-10 minutes, with addition of 40 % PEG on single petri from 10 field of views

Type of fusion	The average incidence (%) \pm SD
Single protoplasts (unfused)	40.69 \pm 14.74
Homofusion between cell mesophyl	4.58 \pm 4.23
Homofusion between cell callus	28.23 \pm 9.44
Heterofusion between cell mesophyl and callus	7.85 \pm 3.70
Multifusion between cell mesophyl and callus	9.69 \pm 4.40

Fused Protoplast Culture

Microscopic observations were done on the phases of fusion protoplasts growth. Protoplasts fusion product grew through several phases in the culture medium, i.e. the formation of cell wall, cell division and cell colony formation. Growth phase in the first week after protoplast fusion is shown in Fig. 3, cytoplasmic hybrid (cybrid) was characterized by the presence of chloroplasts and starch grains in a single cell. The cells are expected to be heterokaryon because nucleus cell will merge so that character of parental cell will combine.

The cell colony development on liquid medium until three weeks after fusion is shown in Fig. 4. Cell colony was bigger and characteristic of cell wall formation was looked clearly. There is a direct relationship between the formation of cell wall and cell division. Cell nuclear division followed by cell divisions and cell division occurs when the cell wall is quite well developed (Bhojwani & Razdan, 1983). Development of cybrid cell to form colonies was expected to produce allotetraploid regenerants. Cell colony from homofusion of callus keprok is expected to be autotetraploid regenerants.

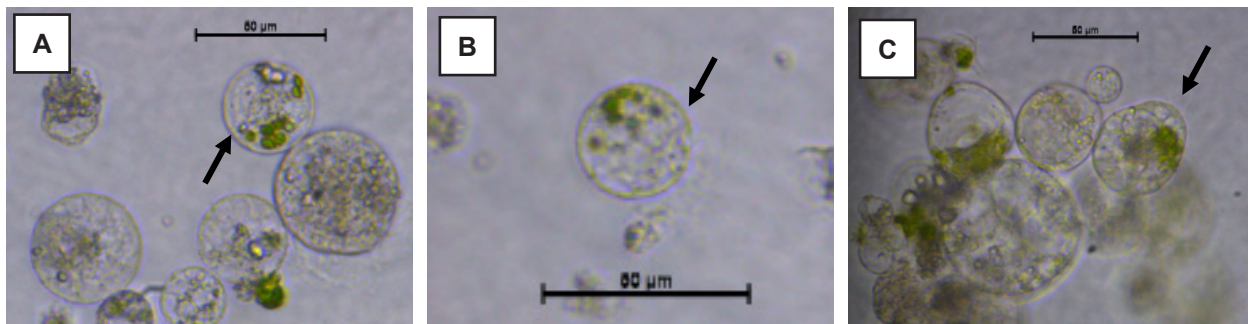


Fig. 3. Protoplasts suspension culture, following pummelo ‘Nambangan’ and keprok ‘Garut’ fusion (1-5 days): (A) One day following fusion (cells began to clump together, and formed cybrids), (B) Cybrids were formed as a result of cell fusion, (C) Five days after fusion, protoplasts regenerating cell wall and underwent cell division. Microscope inverted observation were conducted at 40 magnifications (bar = 50 µm)

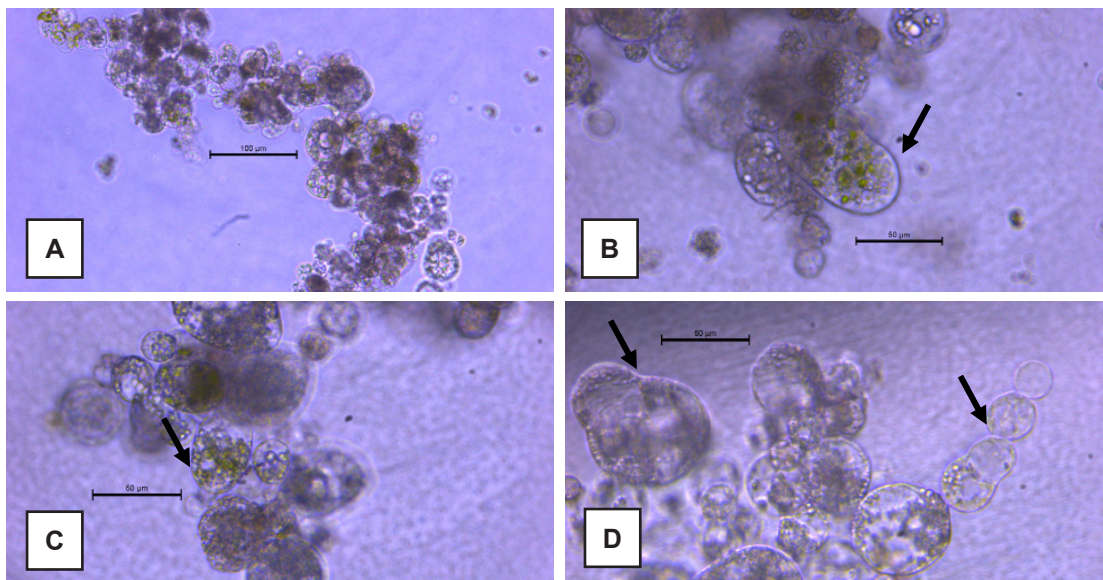


Fig. 4. Pummelo ‘Nambangan’ and keprok ‘Garut’ fusion protoplasts suspension culture after 2-3 weeks: (A) Two weeks after fusion, a colony of cells with about 400 µm long; (B) Two weeks after fusion, cybrid cell was ready to divide (arrow); (C) Three weeks after fusion, cybrid cell was ready to divide; (D) Three weeks after fusion, cell from callus homofusion was ready to divide (arrow). Inverted microscopic observation with 20 magnifications (bar=100µm) for A, and magnification 40 times (bar= 50 µm) for B, C, and D

Observation of the number and diameter of cell colony is presented in Table 3. Protoplast culture from callus keprok was used as control and verified that PEG 40 % is less toxic because two kind of protoplasts resulting cell colony with the similar number and diameter. This is supported by microscopic observation as presented on Fig. 5. However, optimization of the PEG concentration and incubation duration is still needed to obtain higher rate of protoplast fusion product regeneration until forming microcallus (Husni, 2010). Beside that, it is also necessary to optimize the regeneration medium of cell colony for somatic embryo maturation (Widoretno, Indriyani, Martasari, & Hakin, 2017).

Table 3. Number and diameter of cell colony in protoplast culture on 0.4M BH3 medium at 4 weeks' culture

Kind of protoplast	Cell colony density (colony cell.mm ⁻²)	Cell colony diameter (mm)
t Test Significancy	ns	ns
Mesophyl and callus fused	0.20±13.73	0.60±0.16
callus keprok protoplast	0.21±15.83	0.69±0.19

Remarks: ns = not significant

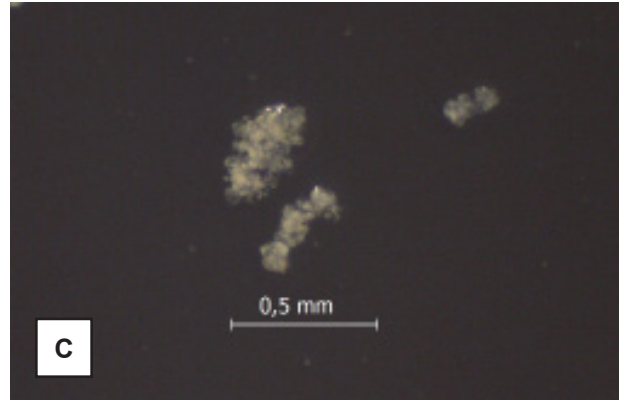
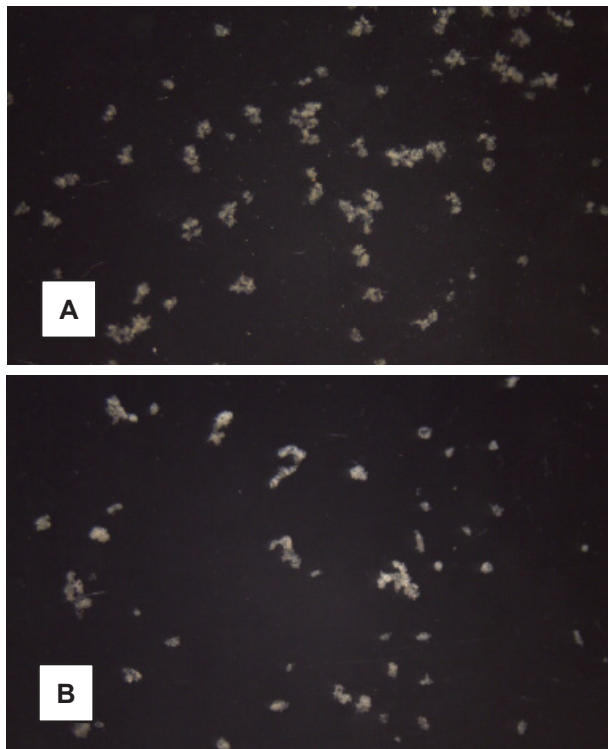


Fig. 5. Protoplast culture of pummelo 'Nambangan' and keprok 'Garut' fusion: Cell colonies from fused protoplast (4 weeks); Cell colonies from callus protoplast (4 weeks); Cell colonies from protoplasts fusion on solid medium (4 weeks). A and B resulted from microscopic observation with 8 times magnification on 0.4 M BH3 liquid medium, C observed with 35 times magnification

CONCLUSION

Isolation of protoplasts from *in vitro* Indonesian pummelo leaves were successfully carried out using a combination of 0.5 % Onozuka cellulase RS10, 0.5 % Macerozyme R10 and 0.1 % Pectolyase Y23 and for embryogenic callus keprok optimum yield was resulted from combination of 0.5 % Cellulase Onozuka RS10 enzyme and Macerozyme R10. Protoplast fusion of pummelo and keprok successfully carried out using PEG mediated method and resulted all protoplasts fused type combination. Cells divided at the first week and began to form cell colonies afterwards in liquid culture.

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