Full Length Research Paper

Optimization of protoplast isolation protocols from callus of *Eurycoma longifolia*

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Protoplast technology offers a unique single cell system that facilitates several aspects of modern biotechnology. In this study, an efficient protocol to isolate the protoplast from callus culture of a valuable medicinal plant in Southeast Asia, *Eurycoma longifolia* was developed. A range of parameters which influence the isolation of *E. longifolia* protoplasts were investigated by using "change-one-factor-at-a-time" method. From the results obtained, callus fresh weight (FW) of 0.2 g produced the highest number of viable protoplasts, which was $1.58 \pm 0.36 \times 10^4$ protoplasts/gFW. The highest amount of viable protoplasts ($1.75 \pm 0.68 \times 10^4$ protoplasts/gFW) was obtained when the sorbitol concentration was maintained at 0.5 M. The optimum enzyme concentration was found to be 1.5% (w/v) of cellulase and pectinase in which $2.75 \pm 1.04 \times 10^4$ protoplasts/gFW were isolated. Meanwhile, an incubation period of 3 h with enzyme solution resulted in the maximum yield of protoplasts ($5.58 \pm 1.46 \times 10^4$ protoplasts/gFW).

Key words: *Eurycoma longifolia*, callus culture, protoplast, osmoticum, viability.

INTRODUCTION

Eurycoma longifolia, from the Simaroubaceae family, is a widely available medicinal plant in Southeast Asia with a long history of use and there is considerable evidence demonstrating its effectiveness in anti-hyperglycemic, anti-malarial, anti-proliferative, anti-schistosomal, anxio-lytic and aphrodisciac (Lin, 2005). Unfortunately, the species is reported to be susceptible to the attack of tiger moth and Sudden Death Syndrome (SDS) in the plantations and in the nurseries (Patahayah et al., 2007). Besides, the growing demands of *E. longifolia* herbal products in the market have led to indiscriminate

collection of the plant as raw material for the drug preparation from the forest floor. Other than that, the distribution of *E. longifolia* in the lowland areas is more prone to over-exploitation of their roots and stems by medicinal plant collectors without proper monitoring and conservative measures. In addition, low efficiency in seed production and germination of *E. longifolia* has also contributed to the gradual disappearance and serious depletion of this plant in nature (Hussien et al., 2006). As the production of a better adapted *E. longifolia* hybrid through conventional plant breeding techniques is difficult and time consuming, crop improvement by biotechnology strategies particularly the somatic hybridization could provide a promising alternative.

The development of protoplast systems has increased the flexibility of plants in biochemical and genetic research (Rao and Prakash, 1995) as well as provides a great prospect in genetic improvement of medicinal plants (Azad et al., 2006). The development of protoplast

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **MS,** Murashige and Skoog.

technology and regeneration procedures played an increasingly significant role in the plant improvement through somatic hybridization and protoplast transformation (Umate et al., 2005). However, a step towards the plant genetic manipulation and integrated approach of breeding programs is primarily laid on an efficient protocol in protoplast isolation, culture and regeneration (Duquenne et al., 2007). Thus, in order to completely explore the application of protoplast-based biotechnology, an efficient protoplast isolation system is necessary. This study aimed to determine the optimum amount of callus, osmoticum and enzymes concentration as well as the incubation period for the maximum yield of viable protoplasts.

MATERIALS AND METHODS

Source tissues

The protoplasts were isolated from the 2-week-old callus cultures of *E. longifolia* maintained on full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) incorporated with 1.0 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, USA), 3% (w/v) sucrose and 0.8% (w/v) agar powder at pH 5.7 ± 0.1.

Standard method of protoplast isolation

The 2-week-old callus was lightly squashed with forceps into smaller and finer cells and transferred into an Eppendorff tube. A total of 400 μL standard protoplast isolation solution which composed of full strength MS medium with 2% (w/v) cellulose Onozuka R-10 (Calbiochem, Germany), 2% (w/v) pectinase (Calbiochem, Germany), and 0.5 M sorbitol (R and M Chemicals, UK) was added. The mixtures were then incubated in darkness for 2.5 hours at 25±1°C under constant agitation (75 rpm) on an orbital shaker (PROTECH model 721, Malaysia).

Standard method of protoplast purification

After 2.5 h of incubation, the mixtures were centrifuged at 1800 g for 3 min. The supernatant was then discarded while the pellet was re-suspended gently with 100 μL of washing medium that consisted of full strength MS medium and 0.5 M of sorbitol. The centrifugation and washing processes were repeated twice.

Determination of viable protoplast

The viability of purified protoplasts was assessed with Evans blue staining and the number of viable isolated protoplasts was estimated using a hemocytometer. The Evans blue staining was initiated by gently re-suspending 15 μL of the protoplasts suspension with 15 μL of 0.4 % (w/v) Evans' blue (Fluka, France). A cover slip was placed over the counting chamber of the hemocytometer. After that, 10 μL of stained protoplasts solution was loaded at each side of the counting chamber through the edge of the cover slip by capillary action. The hemocytometer was placed on the stage of light microscope and the number of viable protoplasts which remained unstained on both sides of counting chamber (8 × 1 mm^2 areas) was counted. The number of viable protoplasts per gram fresh weight was calculated using the formula below:

$$\text{Number of viable protoplast (Protoplast/gFW)}$$

$$= \frac{\text{Average cells} \ \text{x} \ \text{protoplast suspension (in mL)}}{\text{fresh weight (g)}}$$

Optimization of protoplast isolation conditions

The conventional "one-factor-at-a-time" method (Fray and Wang, 2006) was employed to optimize the fresh weight of callus, osmotic condition, enzymes concentrations and incubation time for the protoplasts isolation from *E. longifolia* callus. Only single factor is changed at a time while other factors are kept constant. All procedures for protoplasts isolation and purification were followed based on the standard method described earlier unless otherwise stated.

Fresh weight of callus

The effects of fresh weight of *E. longifolia* callus on protoplast yield were tested at the level of 0.1, 0.2 and 0.3 g. The fresh weight of callus which gave the highest number of viable protoplasts per gram fresh weight (protoplasts/gFW) was fixed and used in subsequent experiments.

Concentration of sorbitol

In this study, the protoplast suspension was purified in washing medium with different concentrations of sorbitol. The effect of sorbitol concentrations on the numbers of viable protoplasts isolated was tested at 0.0, 0.5, 1.0 and 1.5 M. The fresh weight of callus (0.2 g) which yielded the highest number of viable protoplasts in the previous experiment was used in this experiment.

Concentration of digestive enzymes

The effect of different concentration of cell wall degrading enzymes on the number of viable protoplasts isolated was also studied. The combination of 1, 1.5 and 2% (w/v) of both cellulase and pectinase enzymes were added to the protoplasts isolation solution, respectively. The fresh weight of callus (0.2 g) and sorbitol concentration (0.5 M) which yielded the highest number of viable protoplasts were used in this experiment.

Incubation time

In this study, the length of incubation period on the number of viable protoplasts isolated was evaluated. The callus tissues were incubated for 2, 3, 4 and 5 h, respectively, to determine the optimum time required for complete release of protoplasts. The fresh weight of callus (0.2 g), sorbitol concentration (0.5 M) and enzyme concentration (1.5%) which yielded the highest number of viable protoplasts in the previous experiments were used.

Statistical analysis

Each treatment was carried out in three replicates and all the experiments were repeated twice. Variance analysis of the data was carried out and means were statistically compared using
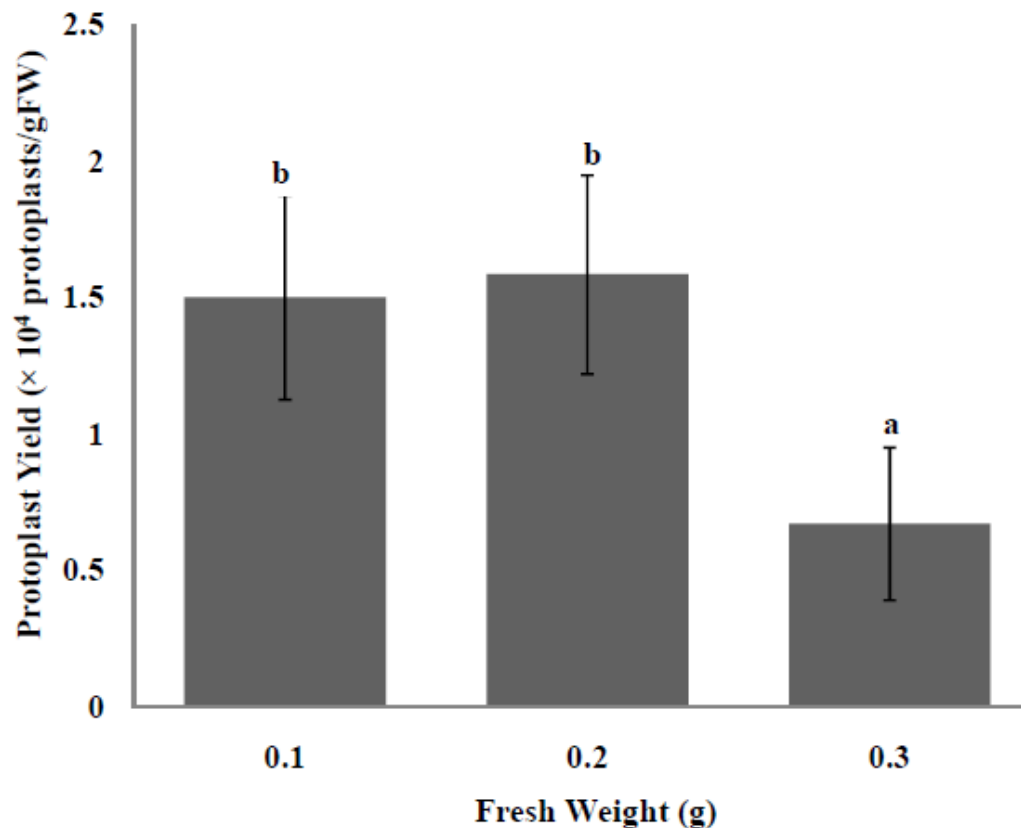
Figure 1. Effects of the fresh weight of *E. longifolia* callus on the number of viable protoplasts isolated. Data represented means ± S. D. of three replicates. Values followed by the same letter are not significant different by Tukey's Honestly Significant Difference (HSD) multiple comparison test at 0.05 probability level.

Tukey's Honestly Significant Difference (HSD) test ($\alpha = 0.05$). Data were analyzed using SPSS analysis (version 15.0) (SPSS Inc.USA).

RESULTS AND DISCUSSION

Effects of fresh weight of callus

The yield of protoplasts was closely dependant on the fresh weight of callus used in the protoplast isolation. The data revealed that the minimum number of viable protoplasts ($1.50 \pm 0.38 \times 10^4$ protoplasts/gFW) was obtained when 0.1 g of callus was used (Figure 1). Meanwhile, the number of viable protoplasts isolated was found to increase apparently by 5.33% to $1.58 \pm 0.36 \times 10^4$ protoplasts/gFW when the fresh weight of callus was increased to 0.2 g. However, a drastic drop in the number of viable protoplasts isolated was observed by nearly one-fold when 0.3 g of callus was employed. Although, the highest number of protoplasts isolated per gram of fresh weight was observed in 0.2 g, there was no significant difference in terms of the protoplasts yield as compared to 0.1 and 0.2 g. Likewise, 0.2 g of friable and

yellow embryogenic suspension cell cultures was chosen to be used in the protoplast isolation of *Cinnamomum camphora* L. (Du and Bao, 2005). In contrast, 4 g of thin callus slices were needed for efficient protoplasts isolation from the woody species *Ulmus minor* Mill. (Conde and Santos, 2006).

In fact, this study was directly concerned with the enzyme-substrate relationship (Bodansky, 1954). In this experiment, the fresh weight of callus was the sole variable and the enzymes concentration in the standard protoplasts isolation solution was the limiting factor. In principle, the cellulase and pectinase enzyme could hydrolyze pectin and cellulose layer of the cell wall of *E. longifolia* callus tissues within a limited area before dissociation of the enzyme occurred (Lenting and Warmoeskerken, 2001). Since the enzyme concentration was constant throughout the experiment, an increase in the fresh weight of callus tissue led to more effective collisions between the callus cells and the enzymes per unit time (Royal Society of Chemistry, 2005). Indeed, a further increase in fresh weight of callus had exceeded the number of active sites that available for the enzymes (Kashyap, 2001). In other words, the enzymes' active sites were virtually used, which had reached the point of
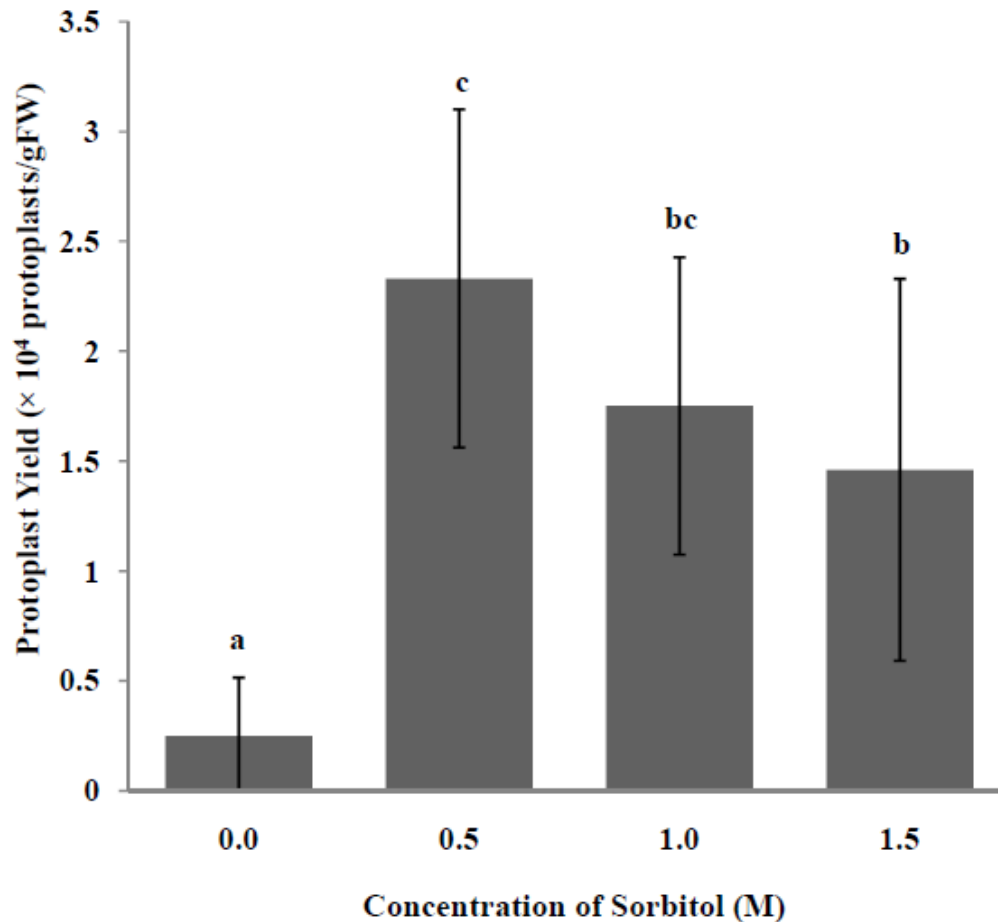
Figure 2. Effects of concentration of sorbitol (M) in washing medium on the number of viable protoplasts isolated. Data represents means ± S. D. of three replicates. Values followed by the same letter are not significantly different by Tukey's Honestly Significant Difference (HSD) multiple comparison test at 0.05 probability level.

saturation and were no longer free to accommodate more callus tissues (Keusch, 2003). The depletion of the enzymes reduced the effective collision between the active site to their targeted substrate when the number of cells per unit volume increased (Nelsestuen and Martinez, 1999).

Effects of concentration of sorbitol

The number of viable protoplasts isolated was strongly affected by the concentration of sorbitol used in protoplasts purification process when the fresh weight of callus was fixed at 0.2 g. The data as illustrated in Figure 2 showed that merely $2.50 \pm 2.65 \times 10^3$ protoplasts/gFW were isolated when sorbital was not added into the washing medium. Meanwhile, a significant improvement of 89.27% in the isolation of viable protoplasts was monitored when 0.5 M of sorbitol was used in the washing medium. Nonetheless, the use of 1.0 M sorbitol led to a decrement of 24.89% in the yield of the viable

protoplasts. Other than that, the number of viable protoplasts dropped abruptly by 16.57% to merely $1.46 \pm 0.87 \times 10^4$ protoplasts/gFW when 1.5 M sorbitol was applied. ANOVA results showed that there was no notably difference in term of protoplast yield between 1.0 and 1.5 M sorbitol. On the contrary, there were significant differences in terms of the cell size and shape when varied osmolarity of sorbitol was used in the washing medium. When the protoplasts were purified without the presence of sorbitol, the protoplasts were swollen and disrupted. Therefore, many non-viable cells and cell debris were observed (Figure 3a). In contrast, a large portion of the viable cells obtained in 0.5 M sorbitol were spherical in shape (Figure 3b). When the protoplasts were purified at higher osmolarity (1.0 and 1.5 M sorbitol), the protoplasts released were shrunken in size (Figure 3c) and lost their spherical shape (Figure 3d).

Generally, the sorbitol acted as flotation agent and sole osmotic stabilizer in isolating a viable protoplast effectively (Jullien et al., 1998). The freshly isolated protoplasts were prone to breakage when sorbitol
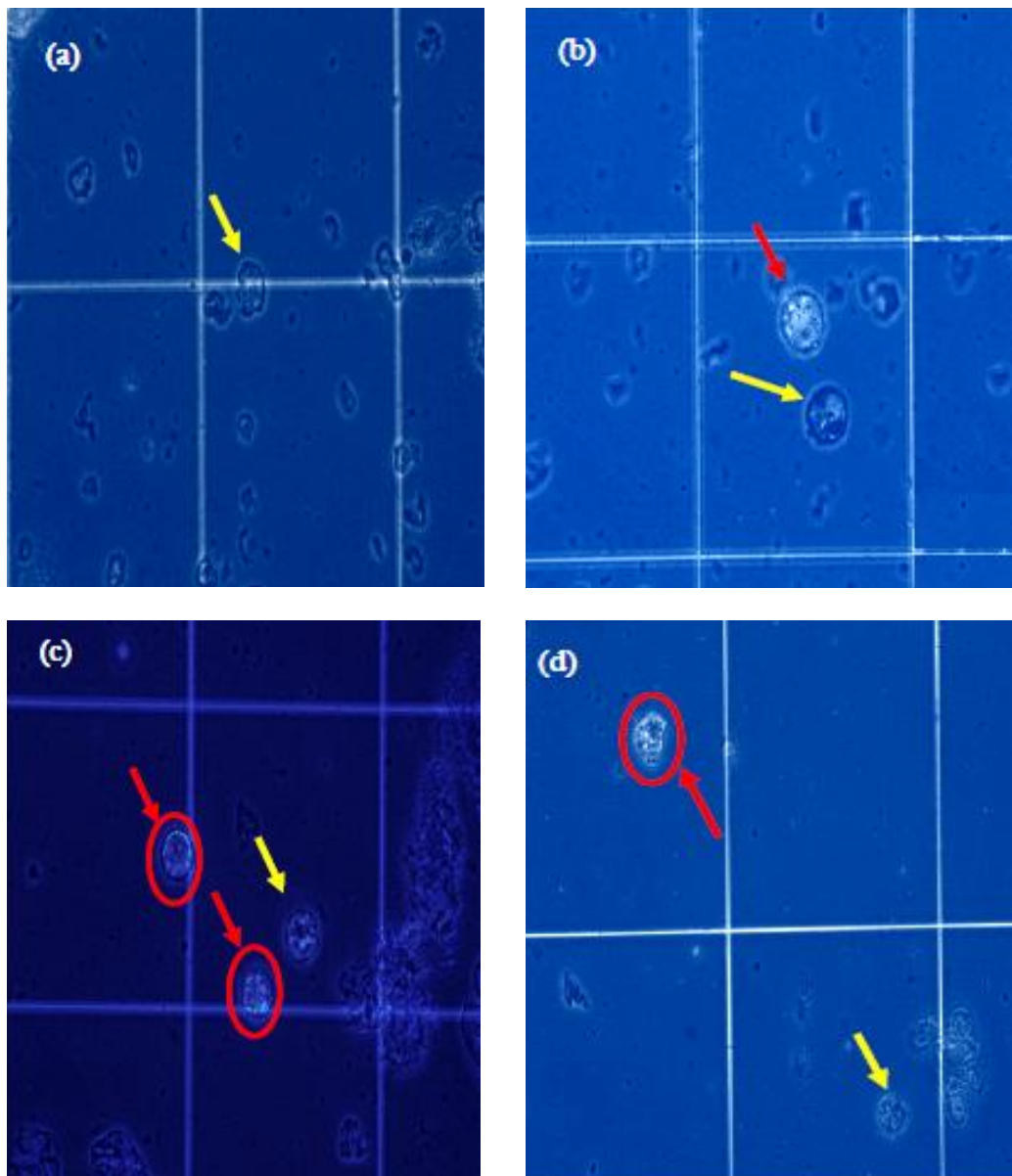
Figure 3. Freshly isolated protoplasts from *E. longifolia* callus in different concentration of sorbitol (200×). (a) 0.0M, (b) 0.5 M, (c) 1.0 M, (d) 1.5 M. Red arrow showed viable protoplasts while yellow arrow showed non-viable protoplasts, which stained with Evans blue dye.

solution was not added to the washing medium. Without osmoticum like sorbital, the water molecules diffused into the protoplasts and caused the cell to rupture (Karp, 2005). A similar result was observed in isolating protoplasts from *Gracilaria verrucosa*, in which no or only few protoplasts were isolated without the presence of osmotic stabilizer (Araki et al., 1998). Conversely, the protoplasts lost their spherical shape when they were purified in the washing medium with sorbitol concentration higher than 0.5 M. A similar result was also demonstrated in the protoplasts isolation of daylilies (*Hemerocallis* spp.) in which the best results were achieved when the osmolority was maintained at 0.7 M sorbitol (Ling and Sauve, 1995). The concentration and type of osmotic stabilizer required for successful protoplasts isolation is varied with the plant species and growing conditions. For example, Sinha et al. (2003) reported that the best yield of protoplasts isolated from *Lupinus albus* L. was optimal at 0.5 M of mannitol. On the other hand, 15% sucrose solution gave the highest number of viable protoplasts in tomato (*Lycopersicon esculentum*) (Tan et al., 1987). Meanwhile, Bass and Hughes (1984) determined that 0.3 M of glucose was sufficient in the isolation of *Elaeis guineensis* protoplasts.
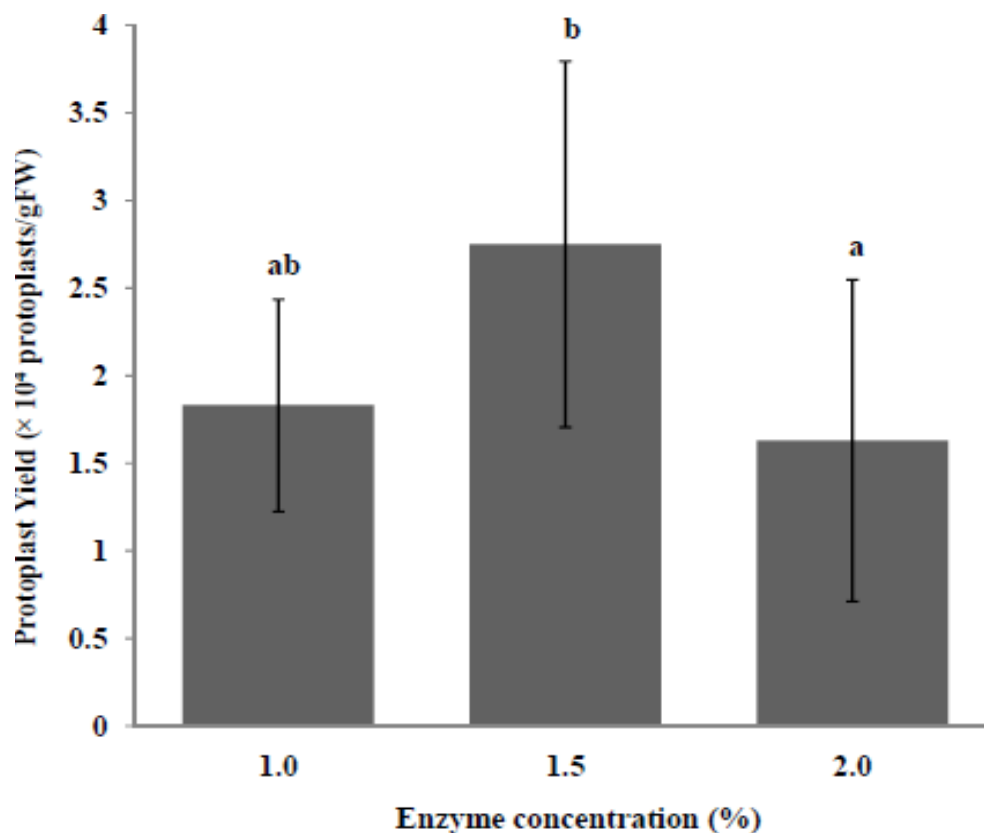
Figure 4. Effects of enzyme concentration (%) on the number of viable protoplasts isolated. The enzyme combinations were maceraseTM pectinase and cellulase onozuka R-10. Values are means ± S. D. of three replicates. Values followed by the same letter are not significant differently by Tukey's Honestly Significant Difference (HSD) multiple comparison test at 0.05 probability level.

Effects of enzymes concentration

The effect of enzyme concentrations on the yield of *E. longifolia* protoplast was demonstrated in Figure 4. The results revealed that the number of viable protoplasts obtained was closely related to the concentration of enzyme used. When the callus tissue was incubated in protoplast isolation solution containing 1.0% of cellulase and pectinase, only $1.83 ± 0.61 × 10^4$ protoplasts/gFW were isolated. Nevertheless, the number of viable protoplasts increased by 33.45% to $2.75 ± 1.04 × 10^4$ protoplasts/gFW and reached the maximum when the enzyme concentration was raised to 1.5%. Indeed, the lowest number of viable protoplasts ($1.63 ± 0.92 × 10^4$ protoplasts/gFW) was obtained when 2.0% of cellulase and pectinase were used. The statistical analysis showed that the protoplasts yield was not significantly different between 1.0 and 1.5% enzyme concentrations even though a higher number of viable protoplasts were obtained in 1.5% as compared to 1.0%. Similarly, notable difference was also not observed in protoplast yield between enzyme concentration of 1.0 and 2.0%. This study was also directly related with enzyme-substrate relationship. In this study, the sole variable was the concentration of cellulase and pectinase enzymes and the callus tissue has become the limiting factor. As the enzyme concentration increased, more active sites were available for effective collisions in the formation of enzyme-substrate complex (Rastogi, 2003). Hence, the number of viable protoplasts isolated was also increased correspondingly. Since the tissues were readily attacked by the enzymes, an increasing enzyme concentration contributed to an increase in the penetration ability of enzymes through multilayer of tightly packed cells in callus (Rao and Prakash, 1995). However, the cellulose and pectin layer of the callus tissues would be saturated with the enzymes in subsequent increases of cellulase and pectinase enzymes to 2.0% (Kremer and Wood, 1992). Therefore, an addition of enzymes per unit volume was unable to further increase the number of viable protoplasts. In contrast, a higher concentration of enzymes has negatively influenced the viability of the protoplasts. The reduction in the yield of viable proto-plasts in excess enzyme concentration was probably due to over-digestion of the protoplasts by pectinase and cellulose enzymes (Raiker et al., 2008).
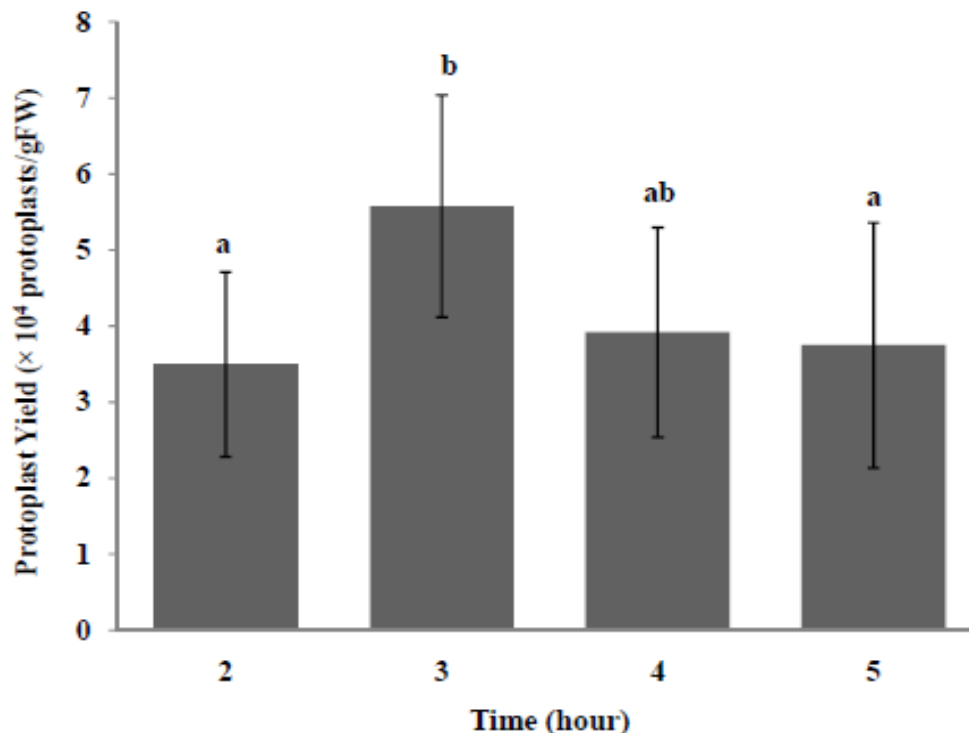
Figure 5. Effects of different incubation periods on the number of viable protoplasts isolated. Data represents means ± S. D. of three replicates. Values followed by the same letter are not significantly different by Tukey's Honestly Significant Difference (HSD) multiple comparison test at 0.05 probability level.

Effects of incubation time

The effects of the incubation time on the number of protoplast isolated were examined when 0.2 g of callus, 0.5 M of sorbitol and 1.5% (w/v) of enzymes concentration were fixed. Figure 5 revealed that the protoplasts yielded in the tissues treated with hydrolytic enzymes increased with the duration of digestion periods but declined with the extended digestion time. In 2 h incubation time, only $3.50 \pm 1.22 \times 10^4$ protoplasts/gFW was isolated. On the contrary, the number of viable protoplasts increased by 37.28% when the exposure period was up to 3 h followed by a slight decline in the yield after 3 h of incubation. The optimum incubation period for high viable protoplast yield from 2-week-old *E. longifolia* callus was 3 h, which yielded $5.58 \pm 1.46 \times 10^4$ protoplasts/gFW. However, a significant reduction in protoplasts yield was observed when the incubation period was increased to 4 and 5 h. For instance, the number of viable protoplasts decreased $3.92 \pm 1.38 \times 10^4$ protoplasts/gFW when the exposure time was 4 h and the yield dropped apparently to $3.75 \pm 1.61 \times 10^4$ protoplasts/gFW in 5 hours digestion period. Although, the highest number of viable protoplasts was obtained in 3 h incubation period, statistical analysis determined that there was no significant difference in terms of protoplasts yield between 3 and 4 h exposure time. Protoplasts yielded after 3 h of digestion periods were significantly difference from 2 and 5 h of incubation.

In this study, the 2 h incubation period was insufficient for digestion of the plant cell materials of the callus tissues and resulted in low protoplast yield. On the contrary, extended incubation period up to 4 and 5 h caused over-digestion of the plant cell which reduced the number of viable protoplasts adversely. Similarly, a high contact of isolated protoplasts to the centrifuge tubes walls in an increased time term of enzyme treatment had eventually reduced the number of viable protoplasts in the protoplast isolation of *Crocus sativus* L. (Darvishi et al., 2006). In contrast, a digestion period of up to 20 h resulted in the best yield of protoplasts (9.45×10^5 protoplasts/gFW) from the callus tissue of the nitrogen fixing woody plant, *Robinia pseudoacacia* (Kanwar et al., 2009).

Conclusions

In conclusion, the isolation of protoplasts from *E. longifolia* callus was best to be carried out by exposing 0.2 g of callus to isolation solution containing 1.5 % (w/v) of cellulase and pectinase for 3 h and further purified with washing medium containing 0.5 M of sorbitol. Nevertheless, other factors such as the age of callus

tissue, the incubation temperature and reaction pH can also be studied. Apart from that, callus can also be induced from the viable protoplast isolated by varying the concentrations and types of plant growth regulators before a successfully plantlet regeneration protocols can be developed.

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REFERENCES

Araki T, Lu Z, Morishita T (1998). Optimization of parameters for isolation of protoplasts from *Gracilaria verrucosa* (Rhodophyta). J. Marine Biotechnol. 6 (3): 193-197.

Azad MAK, Yokota S, Ishiguri F, Yoshizawa N (2006). Plant regeneration from mesophyll protoplasts of a medicinal plant, *Phellodendron amurense* Rupr. *In vitro* Cellular Dev. Biol. – Plant. 42 (6): 502-507.

Bass A, Hughes W (1984). Conditions for isolation and regeneration of viable protoplasts of oil palm (*Elaeis guineensis*). Plant Cell Reports. *3* (5): 169-171.

Bodansky O (1954). Relationship of enzyme concentration to substrate change derived from time-course of reaction. J. Biol. Chem. 209 (1): 281-284.

Conde P, Santos C (2006). An efficient protocol for *Ulmus minor* Mill. Protoplast isolation and culture in agarose droplets. Plant Cell, Tissue Organ Cult. 86 (3): 359-366.

Darvishi E, Zarghami R, Mishani CA, Omidi M, Sarkhosh A (2006). Investigation of the best time of enzyme treatment in order to isolate the protoplast from embryogenic callus to saffron (*Crocus sativus* L.) Biotechnology 5 (3): 284-286.

Du L, Bao MZ (2005). Plant regeneration from protoplasts isolated from embryogenic suspension cultured cells of *Cinnamomum camphora* L. Plant Biotechnol. Reports. *24* (8): 462-467.

Duquenne B, Eeckhaut T, Werbrouck S, Huylenbroeck JV (2007). Effect of enzyme concentrations on protoplast isolation and protoplast culture of *Spathiphyllum* and *Anthurium*. Plant Cell, Tissue Organ Cult*,* 91 (2) : 165-173.

Fray   DD,   Wang   HJ   (2006).   Adaptive   one-factor-at-a-time experimentation and expected value of improvement. Technometrics. *48* (3): 418-431.

Hussien S, Ibrahim R, Ling APK (2006). Adventitous shoot regeneration from root and stem explants of *Eurycoma longifolia* Jack – An important tropical medicinal plants. Int. J. Agric. Res. 1(2) : 183-193.

Jullien F, Diemer F, Colson M, Faure O (1998). An optimizing protocol regeneration of three peppermint cultivars (*Mentha* x *piperita*). Plant Cell, Tissue Organ Cult. 54 (3), 153-159.

Kanwar K, Bhardwaj A, Deepika R (2009). Efficient regeneration of plantlets from callus and mesophyll derived protoplasts of *Robinia pseudoacacia* L. Plant Cell, Tissue Organ Cult. 96 (1) : 95-103.

Karp G (2005). Cell and Molecular Biology. United States of America: John Wiley & Sons.

Kashyap H V (2001). Advanced Topic in Zoology. United States of America: Orient Blacksswam.

Keusch P (2003). Enzyme kinetics*:* Enzymatic decomposition of hydrogen perioxide. Retrieved 25 June, 2009, from http://www.chemie.uniegensburg. de/Organische_Chemie/Didaktik/Keusch/cassylab_kat-e.htm

Kremer SM, Wood PM (1992). Continuous monitoring of cellulase action on microcrystalline cellulose. Appl. Microbiol. Biotechnol.. 37 (6) : 750-755.

Lenting HBM, Warmoeskerken MMCG (2001). Mechanism and interaction between cellulose action and applied shear force, an hypothesis. J. Biotechnol. 89 (2) : 217-226.

Lin KW (2005). Ethnobotanical study of medicinal plants used by the Jah Hut peoples in Malaysia. Indian J. Med. Sci. 59 (4): 156-161.

Ling JT, Sauve RJ (1995). Isolation and culture of daylily mesophyll protoplasts. Plant Cell Reports. 15 (3): 293-296.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15 :473-479.

Nelsestuen GL, Martinez MB (1999). Steady state enzyme velocities that are independent of enzyme concentration: an important behavior in many membrane and particle-bound states. Biochemistry 36 (30) : 9081-9086.

Patahayah M, Lee SS, Farid A., Parid M (2007). Highlights of FRIM's MOSTI Projects 2007: In disease survey of Eurycoma longifolia Jack in peninsular Malaysia. Malaysia: Forest Research Institute Malaysia.

Raikar SV, Braun RH, Bryant C, Conner AJ , Christey MC (2008). Efficient isolation, culture and regeneration of *Lotus corniculatus* protoplasts. Plant Biotechnol. Reports, 2 (3) :171-177.

Rao KS, Prakash AH (1995). A simple method for the isolation of plant protoplasts. J. Biosci. 20 (5) :645-655

Rastogi SC (2003). Cell and Molecular Biology. United States of America: New Age International.

Royal Society for Chemistry (2005). *Enzymes.* Retrieved 16 August, 2009,   from http://www.rsc.org/education/teachers/learnnet/cfb/enzymes.htm

Sinha A, Wetten AC, Caligari PDS (2003). Effect of biotic factors on the isolation of *Lupinus albus* protoplasts. Australian J. Bot. 51 (1) : 103-109.

Tan MC, Rietveld EM, Marrewijk GAM, Kool A.J (1987). Regeneration of leaf mesophyll protoplasts of tomato cultivars (*L. esculentum*): factors important for efficient protoplast culture and plant regeneration. Plant Cell Reports. 6 (3) : 172-175.

Umate P, Rao KV, Kiranmayee K, Sree TJ, Sadanandam A (2005). Plant regeneration of mulberry (*Morus indica*) from mesophyll-derived protoplasts. Plant Cell, Tissue Organ Cult. 82 (3) : 289-293