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Somatic Embryogenesis in Sugar Palm (*Arenga pinnata* Wurmb Merr.) from Zygotic Embryo Explants

Nazatul Asikin Muda[1]* and Asmah Awal[2]

[1]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia
[2]Agricutural Biotechnology Research Group, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia

ABSTRACT

In this paper, a micropropagation protocol of sugar palm (*Arenga pinnata* Wurmb Merr) through callogenesis and somatic embryogenesis was examined. Callus induction frequency and somatic embryogenesis response were dependent on plant growth regulators (PGRs) and genotype. Semi-compact and compact embryogenic calluses were induced from excised immature zygotic embryo (IZE) cultured on semi-solid MS (Murashige & Skoog, 1962) medium supplemented with various concentration and combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl aminopurine acid (BAP). MS medium supplemented with 0.4 mg/L 2,4-D and 0.5 mg/L BAP was found optimum to induce 100% rate of embryogenic calluses and maximum degree of callus formation after 8 and 12 weeks of culture. The incorporation of increased sucrose concentration (60.0 g/L) and 2.0 g/L casein hydrolysate (CH) to the culture medium with similar PGRs composition enhanced the induction of globular somatic embryos (SEs), while addition of silver nitrate (AgNO3) produced SEs of different stages. SEs maturated in MS medium containing 1.0 mg/L BAP and 1.0 mg/L naphthalene-acetic acid (NAA) formed cotyledon-stage embryos. Clonal roots regeneration was obtained on half-strength MS devoid of PGRs after 4 months of culture. Frequent subcultures increased embryogenesis rate favourably.

Keywords: Embryogenic callus induction, immature zygotic embryo (IZE), plant growth regulators (PGRs), somatic embryogenesis, sugar palm

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E-mail addresses:
naza_asyiq87@yahoo.com (Nazatul Asikin Muda),
asmah138@salam.uitm.edu.my (Asmah Awal)
*Corresponding Author

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INTRODUCTION

Sugar palm (*Arenga pinnata* Wurmb Merr) is a multipurpose crop that belongs to the palm species (*Arecaceae*). The communities that live within the rainforests and along their borders have depended on this crop for their livelihood. In the Asian regions, sugar palm

trees have economic importance, being an alternative source of sugar, starch, alcohol and fibres. They are also used in many industrial sectors such as food, beverages, construction, pharmaceutical and crafts (Moore, 2013).

Sugar palm is normally pollinated through seeds and offshoots. However, both seed and vegetative propagation has several limitations including slow fruit development, slow seed germination, non-uniform seedlings, and prolonged dormancy period (Janick & Paull, 2005). Rapid propagation of sugar palm using seeds is not suitable due to heterozygosity and dioecious nature of sugar palm that may result in producing off-type plant. Offshoot propagation is a slow method and not suited for mass propagation due to limited production naturally and the fact that offshoot production of most palm trees is limited to a certain period of their life cycle (Zaid & De Wet, 2013). Therefore, to overcome these propagation problems and to maintain the germplasm, it is beneficial to use the plant tissue culture techniques for multiplication of sugar palm.

Plant tissue culture techniques are used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. The capacity of plant parts or cell to grow into a full plant is termed as "totipotency". Tissue culture techniques are often used for commercial production of plants as well as plant research. Plant tissue culture involves the use of explants, small pieces of plant tissues which are cultured in a nutrient medium under sterile conditions (Smith, 2013). Since the nutrient medium is the only source of nutrition, it should supply all the basic requirements such as carbohydrates, amino acids, minerals, hormones, salts and etc. to cater for plant growth.

Plant growth regulators (PGRs) or plant hormones are organic molecules, critical component in plant tissue culture media that profoundly affect different plant processes such as growth and morphogenesis (Jha, 2005). The five major groups of PGRs i.e. auxins, cytokinins, gibberellins, abscisic acid and ethylene are generally active at very low concentrations and usually work together in coordinating the growth and development of cells, although auxins and cytokinins are by far the most important for plant tissue culturist in determining shoots (high cytokinin-to-auxin ratio), roots (low cytokinin-to-auxin ratio) or callus (relatively high levels of both). The PGRs are also essential to stimulate the development of non-zygotic embryos, growth and development of callus, proliferation of auxiliary roots and development of adventitious roots as well as the establishment of somatic embryogenesis (Beyl, 2011).

Somatic embryogenesis is an efficient approach of plant micropropagation, where embryos are developed from adult somatic cells, known as somatic embryos (SEs). These SEs are bipolar and not connected to explant or callus cells by vascular tissue. While auxins such as NAA and indole-3-butyric acid (IBA) are usually used to induce rhizogenesis on microcuttings, 2,4-D and dicamba (DIC), are commonly used to induce somatic embryogenesis. The ABA is often used to enhance embryo maturation. Somatic embryogenesis has been reported in a number of plant systems which includes palm species (El Bar & El Dawayati, 2014; Kanchanapoom & Phongdara, 2010). Since the SEs are formed without undergoing any fertilisation, they are genetically identical to the parent tissues and are consequently clones. In plant biotechnology, somatic embryogenesis offers numerous benefits mainly at commercial-scale as this system offers an alternative approach to conventional propagation through the unlimited production

of clones with elite traits. Both callogenesis and somatic embryogenesis systems are crucial for plant clonal propagation and are usually an integral part of genetic transformation studies.

SEs induction has been previously reported in sugar palm (Devi, Purwito, & Husni, 2014; Putih, Satria, & Thaib, 2003; Nazatul-Asikin, Awal, Mohd Yusoff, & Shamsiah, 2016). It has been reported that there are some major obstacles in practical application of *in vitro* regeneration method, which may limit the use of this method such as low, irregular multiplication rate of SEs, limited regeneration capacity and loss of totipotency. The present research was designed to evaluate the ideal plant growth regulators (auxin/cytokinin) levels for optimum embryogenic callus induction and somatic embryogenesis of sugar palm for mass propagation throughout peninsular Malaysia.

MATERIALS AND METHODS

Preparation of Plant Materials

Fresh fruits of sugar palm were used as a source of immature zygotic embryo (IZE) explants. Open pollinated bunches were harvested 7-15 weeks after anthesis. Fruitlets were detached from the stalk and soaked in soap water and washed under running tap water. Surface sterilisation was carried out by soaking the fresh fruits in 70% ethanol for 30 minutes, rinsing three times with sterile distilled water and followed by soaking in 50% sodium hypochlorite added with a few drops of Tween 20 for another 30 minutes. After another three sequential rinses with sterile distilled water, plant materials were air dried in laminar air flow chamber (Brand BioBase, BBS-V18000) and cultured on MS medium supplemented with different concentration of PGRs at any possible combinations.

The pH of all media was adjusted to 5.8 prior to autoclaving at 121°C for 20 minutes. The most effective treatment for the induction of callus and embryogenic callus was evaluated. Cultures were then incubated under complete darkness at the temperature of 25±2°C. Periodic subcultures were applied at an interval of four weeks throughout the culture process. Each treatment was replicated 10 times and the experiment was conducted twice. The percentage of callus induction frequency of explant (CIF), average callus fresh weight (ACF), average callus diameter (ACD), its response for degree of callus formation (DCF) and callus morphology were monitored as growth parameters. Results were recorded after 8 weeks of culture.

Somatic Embryogenesis Establishment

Embryogenic calluses at semi globular/globular stage were transferred to SEs maturation medium composed of MS basal medium supplemented with 3%, 4%, 5% and 6% sucrose and casein hydrolysate (CH) at 1.0 – 2.0 g/L. $AgNO_3$ added medium proliferated SEs. Proliferated SEs were maintained in the same culture media until they reach cotyledonary-developmental stage. Maturation of cotyledonary-stage SEs was established in MS medium complemented with 1.0 mg/L BAP and 1.0 mg/L NAA. Cultures were incubated under cool-white fluorescent light (50-60 µmol m^{-2} s^{-1}) at the temperature of 25±2°C and 16-hour's photoperiod. The average number of SEs per explant, ACD (cm), DCF and SEs developmental stages were recorded after 8 weeks of culture.

Somatic Embryogenesis Germination

Culturing 50 SEs determined the germination of SEs and subsequent clonal roots/shoots regeneration at cotyledonary-developmental stage on MS medium supplemented with various compositions of PGRs. Data was recorded based on the parameters of the frequency of clonal shoot regeneration (%) and the frequency of clonal root regeneration (%) for each treatment. MS medium in absence of PGRs (control) served as a control treatment.

Statistical Analysis

The completely randomised design (CRD) was used with 10 replicates for each treatment. Each replicate consisted of one explant. Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) software and the values were expressed as mean ± standard error (SE). The statistical significant difference between means was analysed using analysis of variance (ANOVA) followed by Tukey post-hoc test for comparison between different treatments. Statistical significance was set up at $P<0.05$.

RESULTS AND DISCUSSION

Embryogenic Callus Initiation and Maintenance

Callus culture was established from IZE explants of sugar palm after being cultured in the dark for four weeks at 25°C. The frequency of primary callus induction (CIF) was calculated accordingly as: CIF = (explants forming callus out / total number of embryos being cultured) × 100. Meanwhile, the degree of callus formation (DCF) was classified according to a range of mean size/diameter of the callus formed. Figure 3(a) and Figure 3(b) indicate the growth formation of calluses on IZE explants. In most palm species, high concentrations of auxins particularly 2, 4-D is usually used to initiate callus from zygotic embryo explants (Marbun, Toruan-Mathius, Utomo, & Liwang, 2015). By contrast, in sugar palm, in the presence of high auxin, explants tend to become necrotic and callus tissues growth was hindered.

In general, the primary callus growth was quite slow during the first month of culture. The calluses sizes were very small and the embryogenic callus structures were hardly distinguishable. However, the calluses growth accelerated favourably after 8 weeks of culture and continued to grow in the months after they were accompanied with frequent subcultures. The primary CIF was ranged from 50 to 100% (Figure 1) while ACF (g) and ACD (cm) ranged from 0.28 – 0.68 g and 0.30 - 0.85 cm respectively after 8 weeks of culture. As the callus tissues developed, the colour changed from translucent white to beige or yellowish green. The most optimum CIF (100%) was obtained on the MS medium supplemented with 0.4 mg/L 2, 4-D and 0.5 mg/L NAA with the ACD of 0.850±0.06 cm (DCF: ++) and the AFC of 0.675±0.02 g. Globular-shaped SEs were also detected. While the least CIF was observed on MS + 0.5 mg/L 2,4-D + 1.0 mg/L at 50%, the lowest AFC (0.275±0.02 g) and ACD (0.300±0.09 cm)

were found on MS + 0.2 mg/L 2,4-D + 0.5 mg/L BAP (DCF: +) and MS + 0.3 mg/L 2,4-D + 0.5 mg/L BAP (DCF: +) respectively. The MS devoid of PGRs showed no response for callus development. Statistical analysis using ANOVA and Tukey test showed a significant difference among different treatments ($P<0.05$) (Table 1). The growth response of callus was dependent on the genotype, type and concentrations of PGRs used.

Browning of calluses was also observed on some treatments. Browning of culture is frequently reported in palm species tissue culture system as a result of accumulation of phenolic substances in culture media. In date palm, the lethal browning of explant was prevented by adding activated charcoal to the culture media, followed by frequent subcultures (Al-Khalifah & Shanavaskhan, 2012). Similar corrective measures were also used in oil palm embryogenic callus culture, where it effectively minimized the oxidation and adsorbed toxic phenolic compounds in culture. At the same time, embryogenic callus induction and somatic embryogenesis were also enhanced (Thuzar, Vanavichit, Tragoonrung, & Jantasuriyarat, 2012). In our study, on 2,4-D + BAP added medium, browning was hardly a problem for callus induction, even without the addition of activated charcoal. Frequent subcultures measure also reduced the browning of cultures favourably.

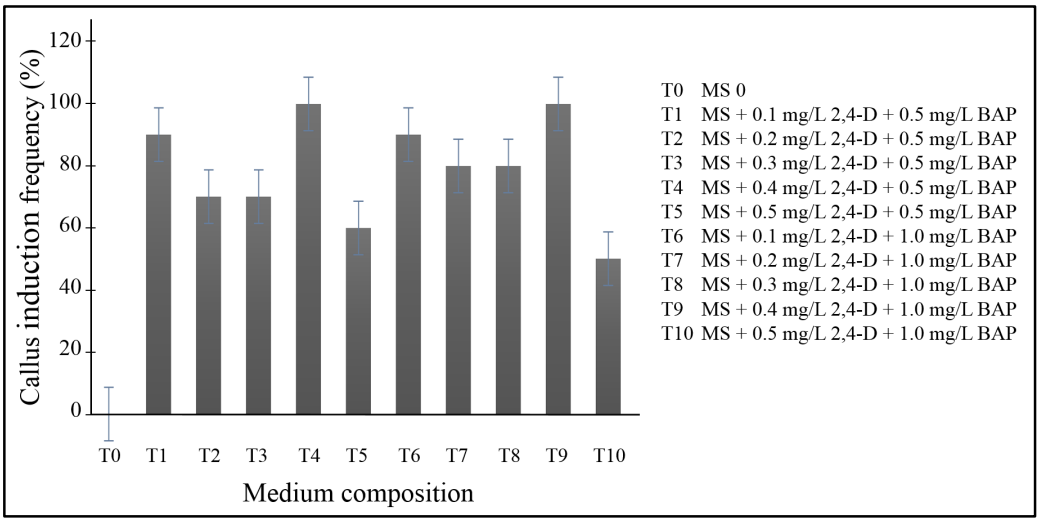

Figure 1. Primary callus induction frequency (%) of IZE explants cultured on MS media supplemented with various concentrations of 2,4-D and BAP after 8 Weeks of Culture. Primary callus induction frequency = (Number of explants inducing callus / Total number of explants cultured) × 100. Error bars indicate the standard error of the mean of ten replicates

Table 1
Statistical analysis on callus development of IZE explants of sugar palm on induction media after 8 weeks of culture

Medium Composition	Average callus fresh weight (g)	Average callus diameter (cm)	Degree of callus formation	Morphology
MS 0	0.000 ± 0.00^{a}	0.000 ± 0.00^{a}	-	No callus
MS + 0.1 mg/L 2,4-D + 0.5 mg/L BAP	0.475 ± 0.05^{bcd}	0.650 ± 0.02^{cd}	++	Compact, translucent, beige callus
MS + 0.2 mg/L 2,4-D + 0.5 mg/L BAP	0.275 ± 0.02^{b}	0.425 ± 0.07^{bc}	+	Compact, translucent, beige callus
MS + 0.3 mg/L 2,4-D + 0.5 mg/L BAP	0.350 ± 0.03^{bc}	0.300 ± 0.09^{ab}	+	Semi-friable, translucent, beige callus
MS + 0.4 mg/L 2,4-D + 0.5 mg/L BAP	0.675 ± 0.02^{d}	0.850 ± 0.06^{d}	++	Semi-friable, translucent, beige callus
MS + 0.5 mg/L 2,4-D + 0.5 mg/L BAP	0.450 ± 0.06^{bc}	0.350 ± 0.08^{bc}	+	Compact, translucent white, beige callus
MS + 0.1 mg/L 2,4-D + 1.0 mg/L BAP	0.288 ± 0.04^{b}	0.425 ± 0.05^{bc}	+	Compact, translucent white, beige callus.
MS + 0.2 mg/L 2,4-D + 1.0 mg/L BAP	0.325 ± 0.05^{bc}	0.350 ± 0.08^{bc}	+	Semi-friable, translucent, beige callus
MS + 0.3 mg/L 2,4-D + 1.0 mg/L BAP	0.375 ± 0.05^{bc}	0.575 ± 0.02^{bcd}	++	Compact, translucent white, beige callus
MS + 0.4 mg/L 2,4-D + 1.0 mg/L BAP	0.525 ± 0.02^{cd}	0.500 ± 0.04^{bc}	++	Semi-friable, translucent, beige callus
MS + 0.5 mg/L 2,4-D + 1.0 mg/L BAP	0.300 ± 0.06^{b}	0.325 ± 0.08^{b}	+	Compact, translucent white, beige callus

[1]Each value within a column is the average of four replications ± standard error (SE). Values followed by the same letter(s) are not significantly different by Tukey-test at $P < 0.05$. [2]Standard error of mean (SEM) was calculated by dividing SD by the square root of sample size (n) [$SE = \sigma/\sqrt{n}$]. [3]Degree of callus formation: i.e. '-', '+', '++', '+++', '++++', '+++++': 'No response', '< 0.50 cm', '0.51 – 1.00 cm', '1.01 – 1.50 cm', '1.51 – 2.00 cm' and '> 2.01 cm' respectively. Data were presented as mean ± SE

Somatic Embryogenesis Establishment

Plant regeneration through somatic embryogenesis occurs in five consecutive steps viz., embryogenic cultures, proliferation of embryogenic cultures, pre-maturation of somatic embryos, maturation of somatic embryos and plant development on medium lacking PGRs (Von Arnold, Sabala, Bozhkov, Dyachok, & Filonova, 2002). Somatic embryogenesis may be direct or indirect in which in the former, somatic embryos are formed directly from explant without the formation of intermediate callus phase. Whereas in indirect somatic embryogenesis, callus is produced first from the explant followed by the regeneration of embryoids from the callus (Slater, Scott, & Fowler, 2003). The initiation of somatic embryos in sugar palm was rather complicated as different steps of induction required addition of various PGRs to the culture medium.

In the present study, somatic embryogenesis proceeded through indirect route as indicated by the induction of intervening callus phase. The SEs were proliferated by transferring the embryogenic calluses to fresh culture medium containing different levels of sucrose (3-6%) and casein hydrolysate (CH) at either 1.0 or 2.0 g/L. Within 4-6 weeks of transfer and frequent subcultures, calluses grew rapidly with matured SEs at semi-globular/globular and heart-shaped stages were detected. The SEs were shiny, beige coloured and grew in clumps (Figure 3(c)). Awal, Taha and Hasbullah (2010) reported optimum direct somatic embryogenesis of *Begonia x hiemalis* Fotsch var. Schwabenland Red on similar culture medium composition.

The average number of maturing SEs per explant, ACD (cm), its formation degree (DCF) and SEs developmental stages after four weeks of transfer are shown in Table 2. Significant difference between all treatments was proved with Tukey test at $P<0.05$ (Table 3). Apparently, pseudoroots development was also observed in MS culture medium with 5% sucrose and 1.0 g/L CH. Similar case of pseudoroots growth was also reported in previous research of sugar palm. It was explained that the formation of pseudoroots in cultures was caused by unstable hormonal balance and its formation was claimed to hinder the formation of clonal sprouts (Devi, Purwito, & Husni, 2014).

The matured clumps of globular SEs were then transferred to elongation medium consisted of similar composition of PGRs for optimum callus induction and 1.0 mg/L AgNO3. After 4-6 weeks of transfer, the somatic embryos elongated and completed the three stages development of somatic embryogenesis (globular, heart-shape, torpedo) [Figure 3d]. Upon transfer to maturation medium containing 1.0 mg/L BAP and 1.0 mg/L NAA, the SEs further developed into clear structures of cotyledonary stage (Figure 3e). The different stages of somatic embryos were observed under light microscope (Olympus, Model CH2oi) and images were captured with a digital camera (Fujifilm FinePix F550EXR).

The developed somatic embryos were then transferred to triiodobenzoic acid (TIBA) added medium for three weeks. The TIBA is an auxin polar transport inhibitor, which inhibits the simulation of *in vitro* lateral root formation (Chen & Chang, 2004) and instead stimulated bud formation (Murashige, 1965). Previous research in sugar palm described the induction and maturation of globular-stage SEs from zygotic embryo explants in the culture media supplemented with high auxin/cytokinin concentration. The authors did not report on further development of the globular SEs after maturation due to high contamination rate, death of explants and limited observation time frame (Devi, Purwito, & Husni, 2014). Nazatul-Asikin (2016) reported optimum induction of embryogenic callus and SEs in sugar palm using basal stem explants after 3 months of culture on MS medium supplemented with low auxin/cytokinin concentration. The report was similar to Wang, Chen, Wu, Lin and Chang, (2013) whom also obtained SEs from the callus of betel nut (*Areca catechu*) cultured on reduced-auxin media.

Table 2
Statistical analysis on the embryogenic callus and somatic SEs of IZE explants of sugar palm responses after 12 weeks of culture

MS Medium Composition (g/L)		Average number of globular somatic embryos per explant	Average callus diameter (ACD) (cm)	Degree of callus formation (DCF)	Somatic embryogenesis developmental stage(s)
Sucrose	Casein hydrolysate (CH)				
30	1.0	17.00±1.99[a]	2.250±0.17[a]	+++++	Semi-globular, globular
40	1.0	26.33±2.33[a]	2.825 ± 0.18[ab]	+++++	Globular, heart-shape
50	1.0	36.33±2.33[a]	3.100±0.16c	+++++	Globular, heart-shape, pseudoroots
60	1.0	29.67±3.93[a]	2.600±0.10[ab]	+++++	Globular
30	2.0	29.00±0.58[a]	2.975±0.23[ab]	+++++	Globular
40	2.0	17.67±1.20[a]	2.525±0.20[ab]	+++++	Globular, heart-shape, semi-globular
50	2.0	65.33±7.69[b]	3.275±0.15[ab]	+++++	Semi-globular, globular
60	2.0	91.33±10.69[c]	3.100 ± 0.35[ab]	+++++	Semi-globular, globular, heart shape

[1]Value of average number of somatic embryos per explant is the average of three replications ± standard error (SE). [2]Value of average callus diameter is the average of four replications ± standard error (SE). [3]Values followed by the same letter(s) are not significantly different by Tukey-test at $P<0.05$. [4]Standard error of mean (SEM) was calculated by dividing SD by the square root of sample size (n) [$SE = \sigma/\sqrt{n}$]. [5]Degree of callus formation: i.e. '-', '+', '++', '+++', '++++', '+++++': 'No response', '<0.50 cm', '0.51 – 1.00 cm', '1.01 – 1.50 cm', '1.51 – 2.00 cm' and '>2.01 cm' respectively. Data were presented as mean ± SE

Somatic Embryos Germination

The optimum germination frequency of SEs of sugar palm was determined on half strength MS medium (½ MS) at 30% (Figure 2). Yellowish green clonal roots with approximate size of 0.5-0.7 cm were visible after four months of culture (Figure 3(f)). No regeneration of clonal shoots/roots of SEs was observed on MS0 and any MS media in the presence of PGRs. Instead, all SEs turned brown and gradually deteriorated. This is contradictory to the result obtained by Abohatem and Baaziz (2011), where they reported a 32% germination rate of date palm SEs on MS medium deprived of PGRs.

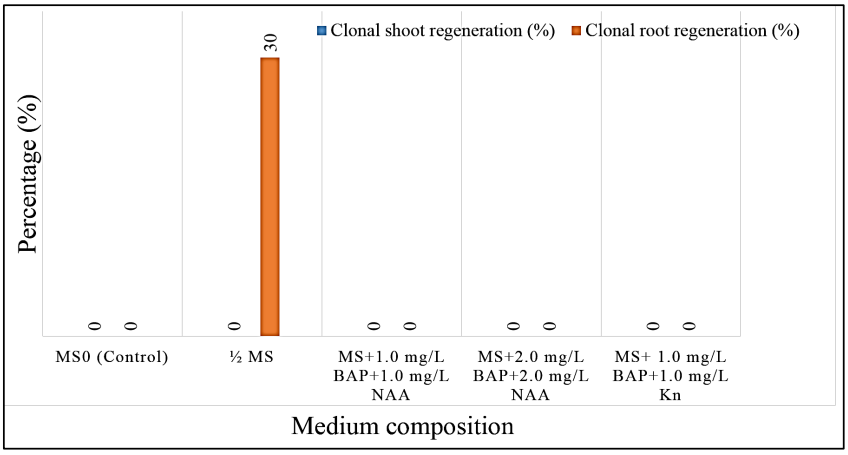

Figure 2. Clonal shoots regeneration and clonal root regeneration frequencies (%) of SEs of sugar palm cultured on different germination media after 4 months of culture. The frequency of clonal shoot regeneration (CSR) and clonal root regeneration (CRR) = Number of explants inducing clonal shoots or roots / Total number of explants cultured) × 100

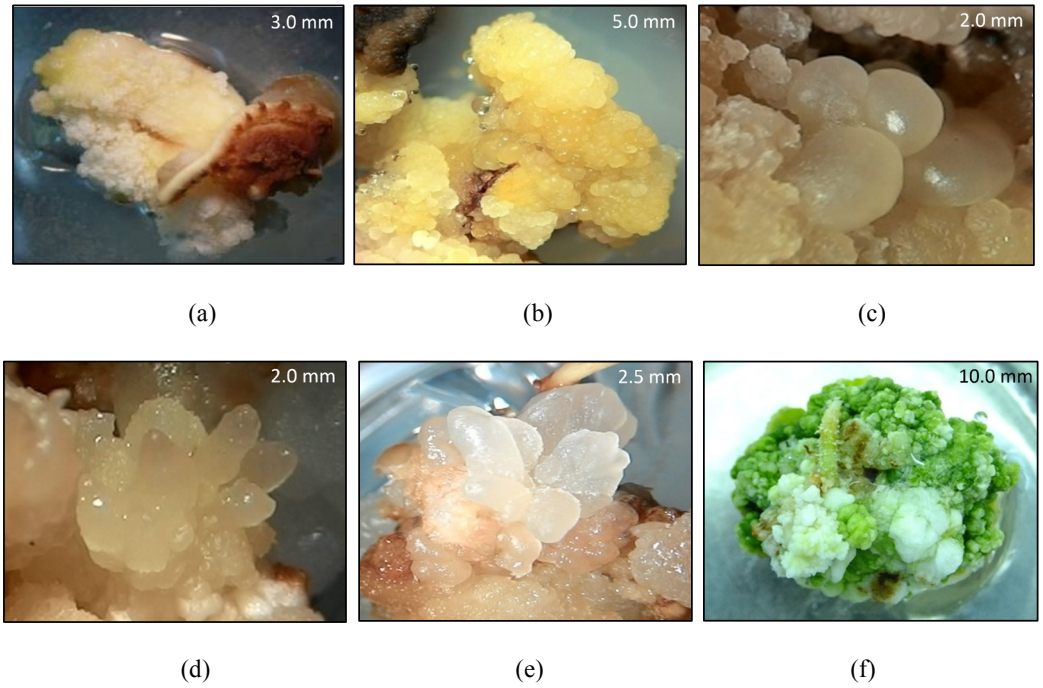

Figure 3. Somatic embryogenesis system from IZE explants of sugar palm (*Arenga pinnata* Wurmb Merr): (a) primary callus induction of IZE explants after 6-8 weeks of culture in culture medium consisted of 0.4 mg/L 2,4-Dichloropenoxyacetic acid (2, 4-D) + 0.5 mg/L benzyl aminopurine acid (BAP); (b, c) progression of globular somatic embryos on primary callus in optimum proliferation culture medium containing 6% sucrose and 2.0 g/L casein hydrolysate (CH); (d) torpedo – stage somatic embryos in clumps on MS + 3% sucrose + 0.4 mg/L 2,4-D + 0.5 mg/L BAP + 1.0 mg/L $AgNO_3$; (e) progression of somatic embryogenesis from cotyledonary stage on MS + 1.0 mg/L BAP and 1.0 mg/L NAA; and (f) clonal root regeneration (arrow) in half-strength MS medium for germination under light condition

CONCLUSION

The present investigation suggested that the embryogenic callus culture from IZE explants is efficient for clonal propagation of sugar palm. Somatic embryogenesis pathway of sugar palm from IZE explants as well as regeneration of clonal roots from maturated somatic embryos (SEs) were successfully determined. However, supplementary investigations to revise this protocol are needed to achieve conversion of cotyledon-stage SEs into complete plantlets for mass propagation and genetic transformation of this species.

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