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Factors affecting haustorium embryo and secondary somatic embryo induction of oil palm (*Elaeis guineensis* Jacq.) 'SUP-PSU1'

Chakriya Niha[1,2], Sompong Te-chato[1,2]* and Sureerat Yenchon[1,2]

[1] *Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand*
[2] *Center of Excellence on Agricultural Biotechnology: (AG-BIO/MHESI), Bangkok 10900, Thailand*

ABSTRACT: Oil palm is an important oil production crop with the high potential oil yield per hectare. Natural propagation of oil palm exclusively occurs by seed. However, oil palm has a single dominant vegetative apex and does not produce adventitious or axillary shoots. Thus, *in vitro* tissue culture is the only means of vegetative propagation. Somatic embryogenesis is one of interesting methods for *in vitro* propagation in oil palm. The objective of this research was to investigate factors affecting haustorium embryo (HE) and secondary somatic embryo (SSE) induction in 'SUP-PSU1' ('25C3/77') oil palm. Embryogenic callus (EC) at 100 mg fresh weight (FW) was cultured on oil palm culture medium (OPCM) or Y_3 (Eeuwens, 1976) medium supplemented with 0.1 mg/L 3, 6-dichloro-2-methoxybenzoic acid (dicamba) or 0.3 mg/L N^6-(2-isopentenyl) adenine (2-iP). The results showed that OPCM supplemented with 0.3 mg/L 2-iP gave the best results in HE induction frequency at 78.11% and average number of HEs at 3.75 embryos/tube. For SSE induction, HE at size of more than 6 mm cultured on MS medium with 0.2 M sorbitol gave the SSE induction frequency at 81.39% and average number of SSEs at 15.10 embryos/response HE after 8 weeks of culture. Therefore, the results can be concluded that HE at size of more than 6 mm induced on OPCM with 0.3 mg/L 2-iP subsequent to transferring to MS medium with 0.2 M sorbitol are suitable protocol for plantlet regeneration of 'SUP-PSU1' oil palm.

Keywords: oil palm; 'SUP-PSU1'; sorbitol; haustorium embryo (HE); secondary somatic embryo (SSE)

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is an important oil producing crop in Southeast Asia, Africa, South America and also very important commercial crop in Thailand especially in Southern Thailand. Palm oil is a very versatile oil as its uses are not limited to food only but also widely used in non-food application such as in detergents, cosmetics, pharmaceuticals and as a feedstock for biofuel (Thuzar et al., 2011). The use and production of biofuels have been strongly promoted in Thailand. In order to achieve a 25 % renewable energy target in 2021, feedstock expansion is needed to satisfy the increased in demand for biofuel production. During 2006–2012, Thailand oil palm showed a rapid increase in plantation area, much higher than that of cassava and sugarcane which are used for bioethanol production (Nilsalab et al., 2017). The architecture of the oil palm, lacking axillary shoot, does not allow for vegetative propagation. Therefore, somatic embryogenesis is the only alternative to seed propagation, which is hampered by long germination times and low germination rate, for the production of planting materials.

* Corresponding author: stechato@yahoo.com

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Somatic embryogenesis is an interesting regeneration pathway for mass and rapid production of oil palm**.** This process defined as somatic cells dedifferentiate into totipotent embryonic cells that can further develop into somatic embryos (SEs) (Guan et al., 2016). In oil palm, the shape of SE obtained is similar to haustorium in monocot plant species. So, called the SE as haustorium embryos (HEs). Many reports revealed that HEs were suitable to use as material for SSE induction (Promchan et al., 2012; Te-chato and Hilae, 2007; Chehmalee and Te-chato, 2008). Both direct and indirect somatic embryogenesis in oil palm has been reported (Hilae and Te-chato, 2005; Te-chato and Hilae, 2007; Chehmalee and Te-chato, 2008; Scherwinski-Pereira et al., 2010; Promchan et al., 2012; Nukoolrat et al., 2016; Sittisak et al., 2017). From those reports, many factors affected somatic embryogenesis in oil palm such as composition of culture media, plant growth regulators (PGRs) and sizes of explant. For culture medium, there are 3 main basal medium that has been used including MS (Scherwinski-Pereira et al., 2010; Thawaro and Te-chato, 2010), Y_3 (Kanchanapoom and Tinnongjig, 2001; Constantin et al., 2015; Jayanthi et al., 2015) and N6 (Sparjanbabu et al., 2018; Thuzar et al., 2011). Whereas oil palm culture medium (OPCM) also reported to use for *in vitro* propagation of oil palm (Kerdsuwan and Te-chato, 2016; Sittisak et al., 2017; Heedchim et al., 2020). Kramut and Te-chato (2010) reported that MS medium gave the highest callus proliferation rate at 90% and globular somatic embryos were started to differentiate after 1 month of culture. Syuhada et al. (2016) revealed that immature embryo (IE) cultured on MS+Y_3 medium gave the highest friable callus formation frequency at 41.25%. Distababjong et al. (2009) reported that embryogenic callus from immature embryo cultured on Y_3 medium with 10 µM NAA and 2 µM abscisic acid gave the highest somatic embryo development at 40.08%. Moreover, exogenous PGRs are the main factor for callus growth and differentiation to SE. Auxins and cytokinins are key regulators of plant cell division and differentiation. In somatic embryogenesis of oil palm, auxins such as picloram, 2,4-D and dicamba are most often used. However, there are few reports about using cytokinin such as 2-iP in somatic embryogenesis of oil palm. 2-iP was reported for somatic embryo induction in many plants such as coffee (Kahia et al., 2016), cassava (Wongtiem et al., 2011) and date palm (Mazri et al., 2018)

Secondary somatic embryogenesis is a process whereby new somatic embryos, so called secondary somatic embryos (SSEs) are initiated from originally formed primary somatic embryos and has certain advantages compared to primary somatic embryogenesis such as very high multiplication rate, independence of an explant source and repeatability. Additionally, embryogenicity can be maintained for long period of time by repeated cycles of secondary embryogenesis (Te-chato and Hilae, 2007). Although secondary somatic embryogenesis was successful in both mono and dicotyledonous plant such as carnation (Karami et al., 2008), mountain ash (Yang et al., 2012), oak species (Martı́nez et al., 2015), physic nut (Loan et al., 2016) and oil palm (Te-chato and Hilae, 2007; Promchan et al., 2012). However, the frequency of SSE induction of oil palm is still low. Therefore, the objectives of this research were to investigate some key factors affecting HE and SSE induction in 'SUP-PSU1' ('25C3/77') oil palm.

Materials and Methods

Plant material

EC used in this experiment was obtained from culturing zygotic embryo of 'SUP-PSU1' ('25C3/77') oil palm at Crop Biotechnology Laboratory, Agricultural Innovation and Management Division, Faculty of Natural Resources,

Prince of Songkla University. Proliferation of the calli was carried out by regular subculture monthly intervals on OPCM (Kerdsuwan and Te-chato, 2016) supplemented with 0.1 mg/L dicamba for 3 months.

All culture media were supplemented with 200 mg/L ascorbic acid, 3% (w/v) sucrose and 0.75% (w/v) agar (Pearl mermaid®). After adjusting pH to 5.7, the medium was autoclaved at 1.05 kg/cm^2, 121 °C for 15 min. The cultures were placed at 26 ± 2 °C under 14 h photoperiod (15 µmol/m^2/s) provided by cool-white fluorescent lamps.

Methods

Effects of culture media and PGRs on HE induction

EC at 100 mgFW was cultured in culture tubes containing 10 mL of various culture media which were modified OPCM or Y_3 medium (Eeuwens, 1976). The media were supplemented with 0.1 mg/L dicamba or 0.3 mg/L 2-iP. After 4 weeks of culture, the percentage of HE induction frequency was calculated using the formula: (number of culture producing HE/total number of culture) x 100, average number of HEs per tube and average number of HEs at different sizes were recorded. A 2X2 factorial design in completely randomized design (CRD) with 5 replications was used and the means among the treatments were separated by Duncan's multiple range test (DMRT) at 1% and 5% probability. The data were statistically analyzed using R program software.

Effect of culture media on SSE induction

HEs at approximate size of 4-6 mm were sorted and cultured in culture tubes containing 10 mL of modified OPCM or MS or Y_3 culture media. All media were supplemented with 0.2 M sorbitol instead of sucrose. After 8 weeks of culture, percentage of SSE induction frequency was calculated using the formula: (number of HE producing SSE/total number of HE) x 100 and average number of SSEs per response HE were calculated. CRD with 5 replications was used and the means among the treatments were separated by DMRT at 5% probability. The data were statistically analyzed using R program software.

Effect of initial sizes of HE on SSE induction

HEs at sizes of 4-6 and >6 mm, were graded and cultured on the best culture medium obtained from the SSE induction experiment. After 8 weeks of culture, percentage of SSE induction frequency was calculated using the formula: (number of HE producing SSE/total number of HE) x 100 and average numbers of SSEs per response HE were recorded. CRD with 5 replications was used and the means between the two treatments were compared by T-test at 5% probability. The data were statistically analyzed using R program software.

Results and Discussion

Effects of culture media and PGRs on HE induction

In the present study, modified Y_3 medium gave the better result in HE induction frequency than OPCM medium (**Table 1**). However, modified OPCM medium gave the higher result in average number of HE per tube than modified Y_3 medium (**Table 2**). So, the results from this present study indicated that OPCM medium is more suitable for HE induction than Y_3 medium. OPCM medium has NH_4NO_3, KH_2PO_4, K_2SO_4 and glycine which lack in Y_3 medium. Those components have been reported to be important for somatic embryo induction in many plants such as cacao (Minyaka et al., 2008), cotton (Haq and Zafar, 2004) as well as in oil palm (Thawaro and Te-chato, 2010; Kerdsuwan

and Te-chato, 2016). Ammonium (NH_4^+) and nitrate (NO_3) are primary sources of nitrogen for plant growth and development. Méndez-Hernández, et al (2019) reported that both nitrate and ammonium content in the culture medium have a significant effect on the response of the explants to the induction of SE. In case of PGRs, 2-iP gave the better results in all parameters than dicamba. For average number of HE per tube and average number of HE at 1-3 and 4-6 mm, significant interaction was found between culture media and PGRs **(Table 2, Table 3 and Table 4)**. After 4 weeks of culture, the highest HE induction frequency at 78.11% **(Table 1)** and average number of HEs per tube at 3.75 embryos **(Table 2)** were obtained from OPCM medium with 0.3 mg/L 2-iP significantly different with other treatments. The new developed HEs were green in color **(Figure 1)** and mostly had sizes of 1-6 mm **(Table 3)**. It has been found that 2-iP played significant role on HE induction in oil palm 'SUP-PSU1'. For somatic embryo induction in oil palm, auxins such as picloram, 2,4-D and dicamba are most often used. However, there are few authors reported the use of cytokinin such as 2-iP in combination with another PGRs for somatic embryogenesis in oil palm. So far, there is no report on the use of 2-iP alone for induction of SE and different results obtained depend on the genotype. Therefore, OPCM medium with 0.3 mg/L 2-iP was suitable for HE induction in oil palm 'SUP-PSU1' ('25 C3/77'). The findings of the present investigation indicated that during the HE induction, PGRs exert greater effect than the culture media. According to this result it suggests that this phase is initial responses due to strong pressure in the cells for cell reprogramming and acquisition of embryonic competence.

Table 1 Effects of culture media and PGRs on HE induction of 'SUP-PSU1' oil palm on different solidified culture media and PGRs with 200 mg/L ascorbic acid after culture for 4 weeks

Culture media	HE induction frequency (%)		Average $^{culture\ media}$
	PGRs		
	0.1 mg/L dicamba	0.3 mg/L 2-iP	
OPCM	13.50±2.92b	78.11±7.09a	45.81±11.36A
Y_3	29.71±5.48b	72.45±6.90a	51.08±8.25A
Average ^{PGRs}	21.61±3.98B	75.28±4.76A	
F $^{(culture\ media)}$	ns		
F $^{(PGRs)}$	**		
F $^{(culture\ mediaxPGRs)}$	ns		
C.V. (%)	27.57		

Data correspond to means±standard error

ns = not significantly different

** significantly different (P≤0.01)

Mean values followed by the same letter within column are not significantly different according to DMRT

Table 2 Effects of culture media and PGRs on average number of HE of 'SUP-PSU1' oil palm on different solidified culture media and PGRs with 200 mg/L ascorbic acid after culture for 4 weeks

Culture media	Average no. of HE (embryos/tube)		Average $^{culture\ media}$
	PGRs		
	0.1 mg/L dicamba	0.3 mg/L 2-iP	
OPCM	1.00±0.00c	3.75±0.56a	2.37±0.53A
Y_3	1.48±0.16bc	2.32±0.17b	1.90±0.18A
Average ^{PGRs}	1.24±0.11B	3.03±0.37A	
F $^{(culture\ media)}$	ns		
F $^{(PGRs)}$	**		
F $^{(culture\ mediaxPGRs)}$	**		
C.V. (%)	30.47		

Data correspond to means±standard error

ns = not significantly different

** significantly different (P≤0.01)

Mean values followed by the same letter within column are not significantly different according to DMRT

Table 3 Effects of culture media and PGRs on average number of HE at 1-3 mm of 'SUP-PSU1' oil palm on different solidified culture media and PGRs with 200 mg/L ascorbic acid after culture for 4 weeks

Culture media	Average no. of 1-3 mm HE (embryos/tube)		Average $^{culture\ media}$
	PGRs		
	0.1 mg/L dicamba	0.3 mg/L 2-iP	
OPCM	0.40±0.24c	1.89±0.33a	1.15±0.31A
Y_3	0.65±0.18bc	1.11±0.08b	0.88±0.12A
Average ^{PGRs}	0.53±0.15B	1.50±0.21A	
F $^{(culture\ media)}$	ns		
F $^{(PGRs)}$	**		
F $^{(culture\ mediaxPGRs)}$	*		
C.V. (%)	43.83		

Data correspond to means±standard error

ns = not significantly different

* significantly different (P≤0.05)

** significantly different (P≤0.01)

Mean values followed by the same letter within column are not significantly different according to DMRT

Table 4 Effects of culture media and PGRs on average number of HE at 4-6 mm of 'SUP-PSU1' oil palm on different solidified culture media and PGRs with 200 mg/L ascorbic acid after culture for 4 weeks

Culture media	Average no. of 4-6 mm HE (embryos/tube)		Average $^{culture\ media}$
	PGRs		
	0.1 mg/L dicamba	0.3 mg/L 2-iP	
OPCM	0.10±0.10c	1.63±0.24a	0.86±0.28A
Y_3	0.79±0.16bc	0.83±0.24b	0.81±0.14A
Average ^{PGRs}	0.44±0.15B	1.23±0.21A	
F $^{(culture\ media)}$	ns		
F $^{(PGRs)}$	**		
F $^{(culture\ mediaxPGRs)}$	**		
C.V. (%)	43.22		

Data correspond to means±standard error

ns = not significantly different

** significantly different (P≤0.01)

Mean values followed by the same letter within column are not significantly different according to DMRT

Table 5 Effects of culture media and PGRs on average number of HE at more than 6 mm of 'SUP-PSU1' oil palm on different solidified culture media and PGRs with 200 mg/L ascorbic acid after culture for 4 weeks

Culture media	Average no. of >6 mm HE (embryos/tube)		Average $^{culture\ media}$
	PGRs		
	0.1 mg/L dicamba	0.3 mg/L 2-iP	
OPCM	0.50±0.22	0.23±0.09	0.36±0.12
Y_3	0.04±0.04	0.38±0.09	0.21±0.07
Average ^{PGRs}	0.27±0.13	0.30±0.07	
F $^{(culture\ media)}$	ns		
F $^{(PGRs)}$	ns		
F $^{(culture\ mediaxPGRs)}$	ns		
C.V. (%)	74.12		

Data correspond to means±standard error

ns = not significantly different

Mean values followed by the same letter within column are not significantly different according to DMRT

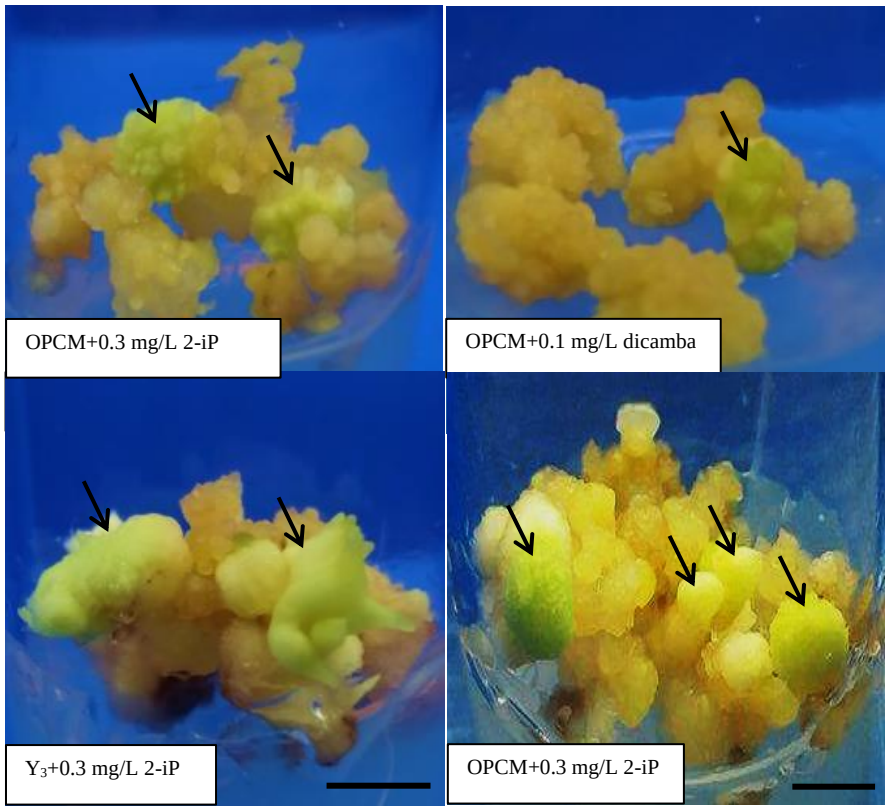

Figure 1 Characteristics of HEs (arrows) derived from cultured EC on different culture media and various types and concentrations of PGRs with 200 mg/L ascorbic acid for 4 weeks (bar = 0.5 cm)

Effect of culture media on SSE induction

After culturing HEs on three different culture media without PGRs supplemented with 0.2 M sorbitol and 200 mg/L ascorbic acid for 8 weeks, the result showed that MS medium gave the significantly highest SSE induction frequency at 23.02% ($P < 0.05$), while OPCM and Y_3 gave the SSE induction at the same frequency of 6.67%. For average numbers of SSEs per response HE, there are not significantly different among the culture media. MS medium gave 7.43 embryos/response HE, followed by OPCM and Y_3 medium which gave 2.00 and 1.50 embryos/response HE, respectively (**Table 6**, **Figure 2**). Similar result was also reported by Te-chato and Hilae (2007) who found that MS medium supplemented with 0.2 M sorbitol gave the highest percentage and number of SSEs. Comparison among three different medium, MS medium containing high concentration of nitrogen compound (NH_4NO_3) and amino acid (glycine) which played on somatic embryo formation. Gomes et al. (2014) reported that the composition of the total free amino acids in somatic embryogenesis, arginine, glutamine, asparagine, alanine, threonine, glycine, serine, proline, leucine and histidine were the most relevant amino acids. Fehe´r et al. (2003), revealed that increase in the levels of total free amino acids in the explant tissues may have occurred because of increased metabolic activity of the cultures lead to physiological and biochemical changes in the growing plant cells. However, the percentage and mean number of SSE formation obtained from the present study was rather low. Thus, MS medium was selected and used for evaluation the effect of sizes of HE on SSE induction.

Table 6 Effect of different PGRs-free solidified culture media containing 0.2 M sorbitol and 200 mg/L ascorbic acid on SSE induction of oil palm SUP-PSU1 culture media with after culture for 8 weeks

Culture media	SSE induction frequency (%)	Average no. of SSEs/response HE (embryos)
OPCM	6.67±6.67b	2.00±0.00
Y_3	6.67±4.08b	1.50±0.50
MS	23.02±2.40a	7.43±1.43
F-test	*	ns
C.V. (%)	87.14	61.57

Data correspond to means±standard error

ns = not significantly different

* significantly different (P ≤ 0.05)

Mean values followed by the same letter within column are not significantly different according to DMRT

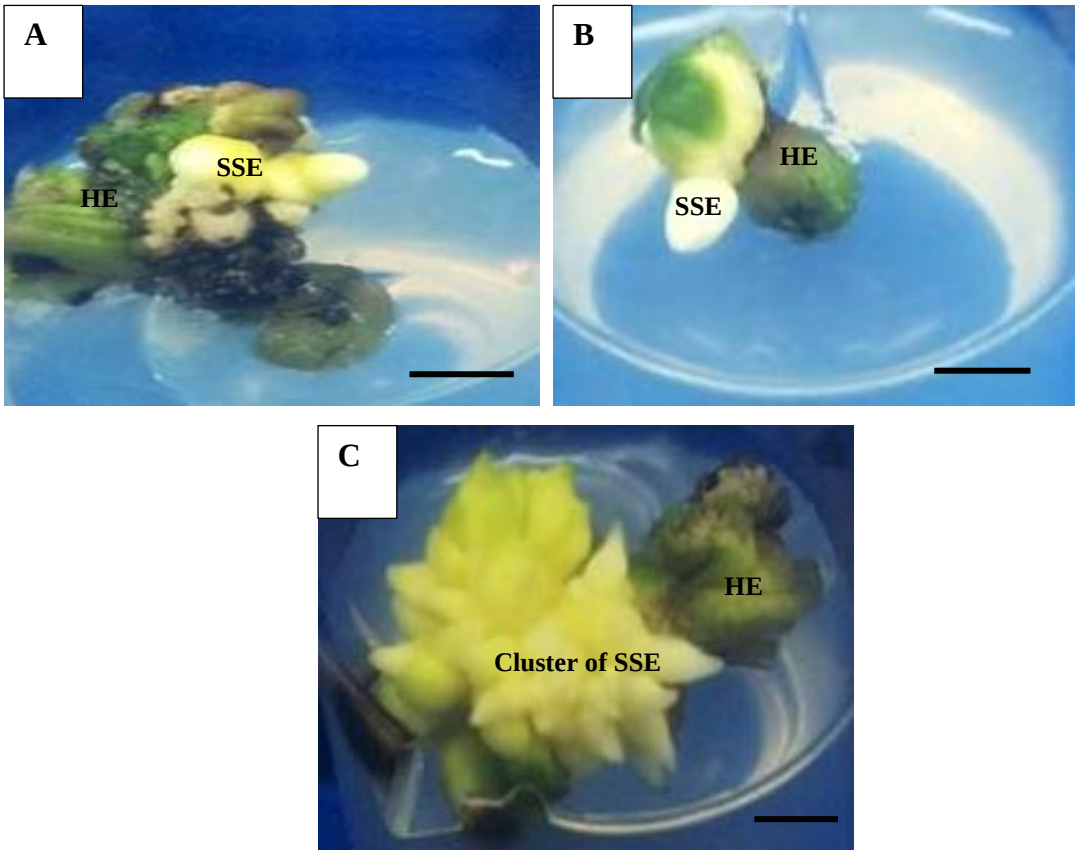

Figure 2 Characteristics of SSEs derived from cultured HEs on different PGRs-free culture media with 0.2 M sorbitol and 200 mg/L ascorbic acid for 8 weeks (bar = 0.5 cm)

A: OPCM B: Y_3 C: MS

Effect of initial sizes of HE on SSE induction

After culturing HEs at different sizes on PGRs-free MS medium with 0.2 M sorbitol and 200 mg/L ascorbic acid for 8 weeks, the result showed that initial sizes of HEs more than 6 mm gave higher SSE induction frequency (81.39%) and average number of SSEs at 15.10 embryos per response HE than those obtained from HEs at size of 4-6 mm (that gave the SSE induction frequency at 59.58% and average number of SSEs per response HE at 11.93 embryos). However, there were not significantly different with those obtained from the two sizes of HEs (**Table 7**, **Figure 3**). Similar result was also obtained from Promchan et al. (2012) who reported that HEs at 13 mm (size ranging from 2-13 mm) gave the highest result in SSE induction in oil palm ('Thepha' clone). Histological analysis demonstrated that SSE of oil palm developed from 3 origins; epidermis, parenchyma and procambium (Promchan et al., 2012). So, the larger size of HE is increasing portion of origin organs that producing SSE and resulting more number of SSEs.

Table 7 Effect of initial sizes of HE on SSE induction of oil palm SUP-PSU1 on solidified PGRs-free MS medium with 0.2 M sorbitol and 200 mg/L ascorbic acid after culture for 8 weeks.

Initial sizes of HE (mm)	SSE induction frequency (%)	Average no. SSEs/response HE (embryos)
4 - 6	59.58 ± 11.35	11.93 ± 0.34
> 6	81.39 ± 6.27	15.10 ± 0.45
T-test	ns	ns
C.V. (%)	29.03	17.67

Data correspond to means±standard error

ns = not significantly different

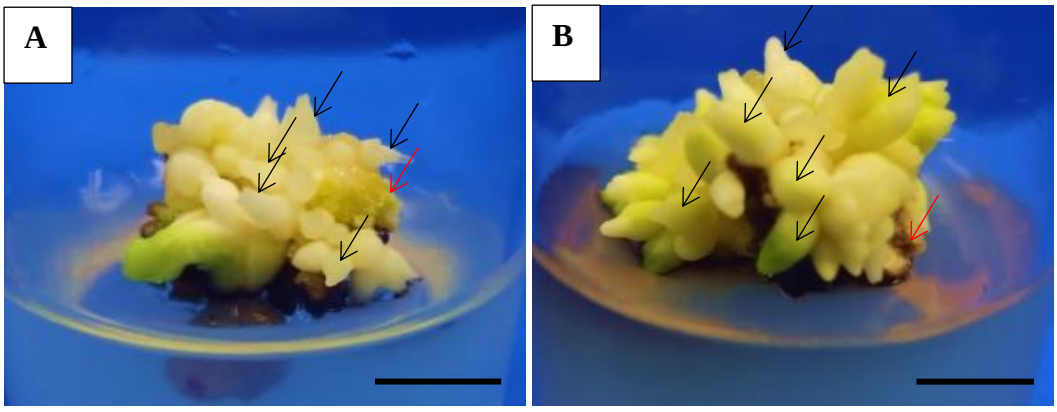

Figure 3 Characteristics of SSEs (black arrows) derived from cultured HEs (red arrows) at different initial sizes on PGRs-free MS medium with 0.2 M sorbitol and 200 mg/L ascorbic acid for 8 weeks (bar = 0.5 cm).
A: 4 - 6 mm B: > 6 mm

Conclusions

EC cultured on OPCM medium with 0.3 mg/L 2-iP gave the highest HE induction frequency at 78.11% and average number of HEs at 3.67 embryos/tube after 4 weeks of culture. For SSE induction, initial size of HE more

than 6 mm cultured on PGRs-free MS medium with 0.2 M sorbitol gave the highest SSE induction frequency at 81.39% and average number of SSEs at 15.10 embryos/response HE after 8 weeks of culture.

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