Induction of oil palm (*Elaeis guineensis* Jacq. var. *Tenera*) callogenesis and somatic embryogenesis from young leaf explants

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ABSTRACT

To determine the optimum medium for callus induction and somatic embryo formation, young leaf explants of oil palm (*Elaeis guineensis* Jacq.) var. Tenera were isolated from 9 and 13 years old trees. Young leaf explants of oil palm (*Elaeis guineensis* Jacq.) var. Tenera were isolated from 9 and 13 years old trees. These explants were inoculated onto solid modified MS and Eeuwens basal media containing 107.41 µM NAA and associated with 0, 22.62, 45.24 or 67.86 µM 2,4-D and onto another MS basal medium with high concentration 450 µM picloram or 450 µM 2,4-D in the presence of 0.03 % (w/v) activated charcoal to induce embryogenic calli. After 28 weeks of culture, compact and pearly-white, globular callus was obtained from the leaf number five (L5). The 2,4-D and NAA media concentration combination (67.86 and 107.41 µM respectively) constituted the optimal medium for embryogenic callus induction, while the media containing only NAA (107.41µM) induced the highest percentage of calli formation (30.56 %). After 36 weeks of culture, direct embryoids were obtained from leaf number six (L6) on the media containing the combination of NAA (107.41 µM) and 2,4-D (45.24 µM). Highest percentage (38.33 %) of direct shoot development was also obtained from the same media NAA (107.41µM).

1. INTRODUCTION

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions 1 often to produce the clones of plants. The resultant clones are true-to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. This tissue culture propagation technique enables mass production of superior clonal palms with high yielding and other desirable traits. Several thousand hectares have been planted successfully in Southeast Asia with clonal oil palms. Clones yielded 9 to 11 t oil/ha from the third year of production onwards, and a world record fruit yield of 50 t fresh fruit bunches (FFB) per ha was recorded in the second year after the start of harvest 2. Currently there are about 21 commercial tissue culture laboratories in the world, involved in oil palm tissue culture, with Malaysia having the largest 3.

In 2009, oil palm clone producers in Malaysia produced 2.53 million ramets. The estimated world production of oil palm tissue culture plantlets is reported for about 3,5 million ramets per year. However, this production of ramets is far below the amount needed by the industry 4. Due to high demand of demand of tissue culture plantlets, oil palm propagation by *in vitro* technique offers a very attractive investment venture in which more than one hundred million of tissue culture plantlets are needed per year 5. The success of many *in vitro* techniques in plants depends on the success of plant regeneration. In fact, the application of some techniques such as *in vitro* mutant and protoplast fusion are suitable to cell culture but may be limited because of the inability to regenerate plants. A vast reservoir of genetic variability is common from *in vitro* plant regeneration 6. Totipotence was first demonstrated by the use of *Nicotiana tabacum* serving as a model for *in vitro* studies 7. The first production of haploid plants by *in vitro* culture of excised anthers was achieved by using *Datura innoxia* 8. The first somatic hybrid was obtained from two *Nicotiana* species, *N. glauca* and *N. Langsdorfii* 9. The initiation and development of embryos from somatic tissues in plant culture was first recognized in the culture of *Daucus carota* tissue 10 11 12.

Oil palm propagation through tissue culture is also widely used. Initially tissue culture techniques were used to propagate elite oil palm clones 13 Several explant sources have been used to perform tissue culture. These include mature embryos 14, inflorescences 15, roots 13, seedlings 16, and young leaves 17 18. Unfortunately some early aberrations were observed from plants produced 19. However, oil palm tissue culture techniques have undergone continuous improvement in recent years. This has resulted in the production of clonal palms with minimal abnormality (less than 5%) 20, 21.

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Moreover, complete plants have been successfully regenerated from various explants of oil palm 22. Although these skills are applied, the frequencies for complete plant regeneration from some explants are still inefficient 23. The low response of embryogenesis is influenced mainly by genotype and donor plant age 24, in addition to the influence of the appropriate media and explants source.

Best explants are from young tissues still undergoing cell division and generally form callus more readily than those from older plant parts, where the meristematic cell activities are reduced or lost. In general, the more juvenile the explant material, the greater the likelihood of success 25, 26. Embryogenesis rates of oil palm usually average less than 5% per ortet 27.

In terms of performance, it was reported 28 that clonal plantlets derived from selected ortets were significantly superior to commercial DxP seedlings. Thus protocol improvements are still needed for tissue culture of oil palm plant propagation. It is in this light that this study was carried out to determine the optimum medium for callus induction and somatic embryo formation from young leaf explants of oil palm of *Tenera* variety. Results of this study could contribute to the development of elite oil palm plant propagation for seedlings production.

2. MATERIALS AND METHODS

2.1 Study site

This work was carried out in the Laboratory of Plant Biotechnology and Tissue Culture, Faculty of Agriculture, Bogor Agricultural University, Indonesia.

2.2 Plant materials and equipment

The starting materials for callus induction were immature leaves isolated from *Tenera* donor palms. The selected trees were about 9 and 13 years old.

Fig. 1: Oil palm young leaf explants.

The donor palm was selected according to some phenotypical characters such as fresh bunch number, height, vigor, and health conditions. The vegetative material samples were collected from Bogor Agricultural University Experimental Station of Cikabayan oil palm plantation. The explants used in this study are shown in Figure 1.

2.3 Media preparation

Two different modified basal nutrient media, Murashige and Skoog medium 29 and Eeuwens and Blake medium 30, containing macro and micro salts (Table 1&2), were used for callus induction and embryo proliferation. These media were supplemented with NAA, glutamine, asparagine, 2,4-D and Picloram.

Table 1: Murashige and Skoog (MS) medium composition used in the experiment.

Stock	Compound	Stock solution concentration (g/l)	Pipette volume (ml/l media)	Media concentration (mg/l)
A	NH_4NO_3	82.500	20	1 650
B	KNO_3	95.000	20	1 900
C	KH_2PO_4	34.000	5	170
	H_3BO_3	1.240		6.2
	$Na_2MoO_4.2H_2O$	0.050		0.250
	$CoCl_2.H_2O$	0.005		0.025
	Kl	0.166		0.830
D	$CaCl_2.2H_2O$	88.000	5	440
E	$MgSO_4.7H_2O$	74.000	5	370
	$MnSO_4.4H_2O$	4.460		22.3
	$ZnSO_4.7H_2O$	1.720		8.6
	$CuSO_4.5H_2O$	0.005		0.025
F	$Na_2EDTA.2H_2O$	3.730	10	37.3
	$FeSO_4.7H_2O$	2.780		27.8
VIT	Thiamine	0.010	10	0.1
	Niacin	0.050		0.5
	Pyrotoxine	0.050		0.5
	Glycin	0.200		2.0
Myo	Myo inositol	10.000	10	100

Source: [29]

Table 2: Eeuwens and Blake medium (Y_3) composition.

Stock	Compound	Stock solution concentration (g/l)	Pipette volume (ml/l media)	Media concentration (mg/l)
A	NH_4Cl	26.750	20	535
	KCl	74.600		1492
B	KNO_3	40.400	20	2020
C	$NaH_2PO_4.2H_2O$	55.200	5	276
	H_3BO_3	0.620		3.1
	$Na_2MoO_4.2H_2O$	0.048		0.24
	$CoCl_2.6H_2O$	0.048		0.24
	Kl	1.660		8.30
	$NiCl_2.6H_2O$	0.0048		0.024
D	$CaCl_2.2H_2O$	58.800	5	294
E	$MgSO_4.7H_2O$	49.400	5	247
	$MnSO_4.H_2O$	2.240		11.2
	$ZnSO_4.7H_2O$	1.440		7.2
	$CuSO_4.5H_2O$	0.050		0.25
F	$NaEDTA.2H_2O$	3.720	10	37.2
	$FeSO_4.7H_2O$	1.390		13.9
VIT	Thiamine	0.050	10	0.5
	Niacin	0.005		0.05
	Pyrotoxine	0.005		0.05
	CaP	0.005		0.05
	Biotin	0.005		0.05
Myo	Myo inositol	10.000	10	100

Source: [30]

2.4 Sterilization compounds

Standard sterilizing agents such as alcohol (5%; 10% and 70%), Sodium hypochlorite (bleach) (5.25% NaClO), detergent, fungicide (Dithane M-45 2g/l, Agrept 2g/l) and antibiotic (Amoxicillin 1g/l), aquades steril were used in the experiment.

2.5 Laboratory equipment and tools

The equipment and tools used in the experiment are pipette fillers (1 ml and 10 ml), autoclave 2 l, wrapping plastic, 250 and 270 ml glass bottles (baby food jar), magnetic stirrer, magnetic shaker, digital analytical scale balance, bunsen burners, erlenmeyer volumetric flask (250, 500 and 1000 ml), graduated cylinders (100 and 500 ml), hot plate**,** laminar air flow cabinet, spatula, pincer, sterilized blades, scalpel (knife), forceps, pH meter, Petri dishes, thermometer, pot holder, refrigerator, gas ring, labeler, cup holders (1000 and 2000 ml), towel, papers, sprayer, disposable mask, and disposable hand gloves.

2.6 Methods

2.6.1 Oil palm tree sampling and explant processing

The donor palm used for explants isolation was a 9 years old Tenera variety. After the palm was identified, the spear was carefully removed by using a curved knife and with the help of a specially designed protocol to ensure that the donor palm and the candidate spear are not damaged during the isolation process. As soon as the spear was isolated from the palm, it was sprayed with 96% alcohol, wrapped and placed inside a sampling bag. The protected spear (Figure 2a) was then taken immediately to the laboratory for processing.

The spear is a cluster of unopened young leaves or fronds which differ in age (Figure 2b). The leaves were carefully removed from the spear in laminar air flow cabinet of the dissecting or plant preparation room and grouped per cluster (Figure 2d). The leaves were then soaked in a 10 % NaOCl (household bleach) solution for 10 minutes (sterilization) and rinsed three times in sterile distilled water. Each cluster of leaves was then soaked in 10% sterile sucrose solution.

Leaves were sliced into 1 cm length and inoculated onto callus induction media using four explants per vessel. From one spear, around 3600 pieces of explants were extracted.
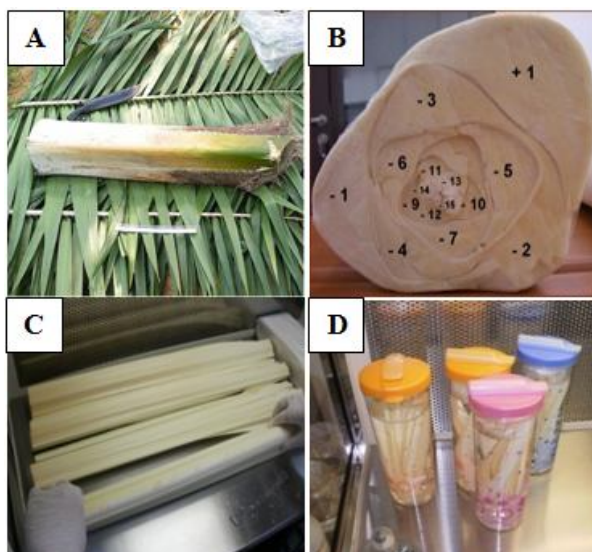
Fig. 2: Young leaf samples processed from the field to laboratory.
A. Isolated spear, B. Young leaf clusters in the spear, C. Isolated cluster of young leaves, D. Young leaves soaked in sucrose solution after sterilization.

2.7 Callogenesis mixing medium

Ten treatments were applied: C1, C2, C3, C4, C5, C6 for Murashige and Skoog medium and M1, M2, M3, M4, for Eeuwens and Blake medium (Y_3). The tested media compositions were as follow:

C1 = MS + 0 µM 2,4-D + 107.41µM NAA
C2 = MS + 22.62 µM 2,4-D + 107.41µM NAA
C3 = MS + 45.24 µM 2,4-D + 107.41µM NAA
C4 = MS + 67.86 µM 2,4- D + 107.41µM NAA
C5 = MS + 450 µM picloram
C6 = MS + 450 µM 2,4-D
M1 = Y3 + 0 µM2,4-D + 107.41µM NAA
M2 = Y3 + 22.62 µM 2,4-D + 107.41µM NAA
M3 = Y3 + 45.24 µM2,4-D + 107.41µM NAA
M4 = Y3 + 67.86 µM 2,4 D + 107.41µM NAA

The pH of nutrient media (C1, C2, C3, C4, M1, M2, M3, and M4) was adjusted to 5.7 with 1N NaOH or HCl before the purified agar was added and autoclaved. For each medium, 5% of commercial sugar and 0.9% of purified agar to make solid medium were used. For C5 and C6 media, 3% of commercial sugar was used while the pH was adjusted to 5.8 before adding 0.3 g/l charcoal and 0.9% of purified agar. The Media were dispensed into culture jars (250 ml) and covered with heat resistant plastic. Then media were autoclaved for 20 minutes at 121 ^{o}C and 0.1 bar (17.5 psi).

The experimental design was a factorial randomized complete block design with 3 replications. Each treatment was applied in 30 jars so that a total of 960 explants could be used per replication. The first factor considered was the rank of leaf with 5 levels (L-5, L-6, L-7, L-8, L-9) and the second factor was the concentration of the tested growth hormone with 10 levels (C1, C2, C3, C4, C5, C6, M1, M2, M3, M4). Another factor, the type of basal media solution with two levels (MS and Eeuwens media) was also taken into account.

2.8 Environmental culture conditions

Cultures were incubated under darkness to induce callus in a temperature-controlled room at 28 ± 1 ^{o}C. Observations were done every week for the percentage of contamination and every four weeks for the percentage of explant developing callus, the percentage of embryogenic callus, and the percentage of embryoid. Qualitative variables recorded were the color of explant *(browning)*, the color of callus, and the callus structures (nodular, root-like, and embryogenic). As soon as calli were initiated, they were transferred in the new same media composition. The subculture of the initial explant for callus initiation was done at 12 week intervals, while the initiated callus was subcultured at 8 week intervals.

2.9 Culture conditions of embryos

The embryoid stage marks the beginning of the light phase of the oil palm tissue culture process. The embryogenic cells and embryoid cultures were transferred from continuous dark

culture to continuous light culture room for incubation with more light intensity of 1000 lux at 29 ± 1°C. The number of embryo cells was recorded.

2.10 Embryogenesis mixing medium

Calli are undifferentiated tissues which have the ability to give rise to unspecialized cells (embryogenic Calli) that are meristematic and can produce embryoids under good conditions. As soon as embryogenic calli or embryoids were produced, they were transferred to embryoid media for their proliferation or multiplication and maturation. These media consisted of the same basal medium composition but with 25% of gradually reduced concentration of 2,4-D and NAA from the 3rd to 4th subcultures. Each subculture was done after 6 week intervals. The flow chart of subcultures is given in figure 3.
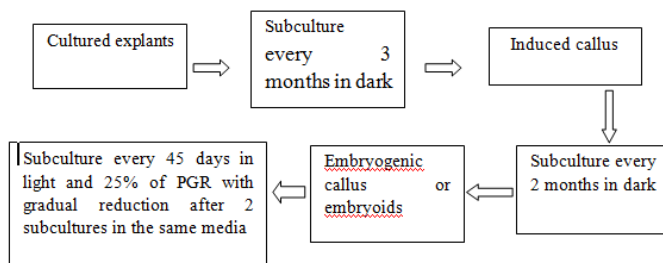

Fig. 3: Subculture flow chart of the experiment.

2.11 Data Analysis

The ANOVA of data obtained was done using Excel software. The means were separated with Student-Newman-Keuls tests at the 0.01 and 0.05 levels of probability, respectively. The F-test was used to show significant differences among means.

3. RESULTS AND DISCUSSION

3.1 Callus induction of young leaf explants

The leaf explants were swollen after 2-3 weeks in callus induction culture medium. It has been reported 31 that callus production is always extremely slow for palms. This assertion was in harmony with our result. In fact callus induction was first observed from young leaf number nine (L-9) after 8-12 weeks of culture on medium C1 containing only NAA (107.41μM) as plant growth regulator.

These calli appeared at the top of explant surface on the vascular region as shown in Figure 4. Another type of callus was observed. It consisted of hard root-like callus which appeared to develop from the tips of explants as shown in Figure 5. Calli were grey to dark-grey and white in color and had a friable consistency. After the 39th week, callus production varied from 1.39% to 30.56% depending on the combination of NAA and 2,4-D; the rank of leaf and subsequently the type of basal media (Table 3). A similar type of callogenesis was reported by 32 in zygotic embryo of oil palm. C1 media gave the best percentage of explant bearing callus, and the young leaf rank (L-9) was more responsive than older leaf explants (L-5, L-6, L-7, and L-8). From the obtained

calli, 87% were induced from MS media and the other 13% were observed from Eeuwens media.
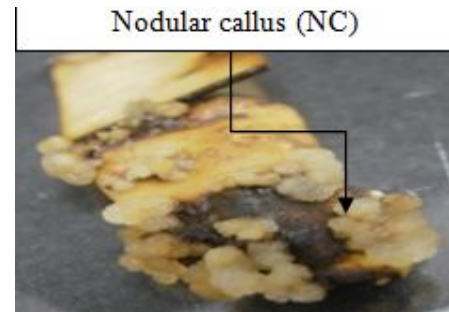

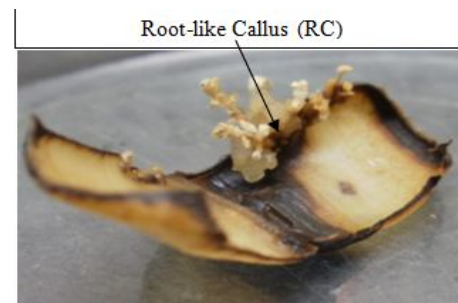**Fig. 4:** Friable NC proliferation from young leaf explants 24 weeks after inoculation.

Fig. 5: Root-like callus initiation from young leaf explant after 24 weeks

According to the statistical analysis, it was observed in this study that there is a very highly significant influence of the type of medium concentration (Table 4). The C1 media composed of MS as basal medium and NAA as plant growth regulator was optimal for callogenesis response from immature leaf explant of oil palm (Table 5). This result showed that the presence of NAA only at the concentration of 107.41μM in MS basal medium promoted callus initiation and proliferation. However, by adding 2,4-D in the same media, the ability of meristematic cells activity to induce callogenesis was reduced. Another worker 33 reported that, auxins have multiple roles in tissue culture according to their chemical structure, concentration, and the plant tissue being affected. Auxins influence callus production, cell elongation, and cell division in cambium tissue. No significant difference was observed between the leaf ranks of oil palm (*Elaeis guineensis* Jacq.) var. *Tenera* at the threshold α = 0.05 for callus induction (Table 6). This result showed that all the young leaf ranks (L-5, L-6, L-7, L-8, L-9) of the spear gave the same response for callogenesis and can be easily exploited for callus induction purpose in embryogenesis experiments. In other words, the young leaf cells from the spear are potentially totipotent, competent to receive the signal that triggers the callogenesis pathway. It was reported that, the presence of auxin in the earlier age of inflorescence explants stimulated callus induction 0. According to 22, the success in obtaining callus formation was likely due to the use of very young explants, the presence of suitable concentration of the auxin, and strict subculture regime.

Table 3: Influence of various medium composition (NAA and 2,4-D) and young leaf rank of oil palm (*E. guineensis*) cv.Tenera spear on the percentage of explant bearing callus after 39 weeks.

Rank of leaf on the spear	Type of medium composition									
	MS medium						Eeuwens medium			
	C1	C2	C3	C4	C5	C6	M1	M2	M3	M4
	Percentage (%) of callogenesis									
L-5	11.11	0	0	2.78	0	0	1.39	0	0	0
L-6	1.39	0	0	0	0	0	0	0	0	0
L-7	6.94	0	0	1.39	0	0	0	0	0	0
L-8	12.50	0	0	0	0	0	1.39	0	0	0
L-9	30.56	0	0	0	0	0	6,94	0	0	0

Note: C1-4 = MS+(0, 22.62, 45.24, 67.86µM) 2,4-D+107.41µM NAA, C5= MS+450µM picloram, C6 = MS+450 µM, M1-4 = Y3+(0, 22.62, 45.24, 67.86µM) 2,4-D+107.41µM NAA. L-x = leaf number in the spear.

Table 4: NOVA of callus induction from immature leaf explant of oil palm

Source	DF	Type III SS	Mean Square	F Value	Pr> F
Replication	2	28.84	14.42	3.60	0.0310
Medium	9	196.37	21.82	5.45	<.0001
Leafrank	4	12.71	3.18	0.79	0.5324
Medium*leafrank	36	71.03	1.97	0.49	0.9910
Error	98	392.49	4.005		
Corrected Total	149	701.44			

Table 5: Mean number of young leaf explants of Tenera oil palm bearing callus per media

Type of medium composition	Mean number of callogenesis per media
C1	3.86 a
C2	0 b
C3	0 b
C4	0,40 b
C5	0 b
C6	0 b
M1	0.53 b
M2	0 b
M3	0 b
M4	0 b

Note: Means with the same letter are not significantly different according to Student-Newman Keuls test (P<0.05). C1-4 = MS+(0, 22.62, 45.24, 67.86µM) 2,4-D+107.41µM NAA, C5=MS+450µM picloram, C6 = MS+ 450 µM, M1-4 = Y3+(0, 22.62, 45.24, 67.86µM) 2,4-+107.41µM NAA

Table. 6: Effect of the spear leaf rank of oil palm var. Tenera on callogenesis after 39 weeks in medium

Rank of leaf on the spear	Mean number of callogenesis per leaf
L-5	0.70 a
L-6	0.07 a
L-7	0.40 a
L-8	0.33 a
L-9	0.90 a

Note: Means with the same letter are not significantly different with Student-Newman-Keuls test (P<0.05). L-x = leaf number in the spear.

3.2 Embryogenesis of young leaf explants

After approximately 28 weeks of culture, a small, compact and pearly-white, globular callus was observed in culture derived from the leaf number five (L-5) (Figure 6). As compared to other young leaves, L-5 is the oldest from the isolated spear and was competent to initiate embryogenic callus. These pearly-white cells were obtained on C4 media containing MS basal medium supplemented with the combination of NAA (107.41 µM) and 2,4-D (67.86 µM). The success in obtaining embryogenic callus was likely due to the use of potential competent young leaves (L-5) which in the presence of suitable concentration of 2,4-D (67.86 µM) and strict subculture regime, triggered the embryogenic induction pathway. Similar cell structures were reported in immature inflorescences of coconut 35 and in immature

inflorescences of the oil palm [22]. From all the obtained calli, just 3.63% of embryogenic calli were produced. Other workers 36 observed that the crossing type of plants or genotypes play an important role in somatic embryogenesis. After 36 weeks of culture, another different mass cell was observed from explants. It consisted of direct big, compact and pearly-white globular callus (direct embryoid) obtained from leaf number six (L-6) on the C3 medium containing the combination of NAA and 2,4-D (Figure 7).

From that mass tissue 15 single cells were obtained. In fact, the increase of 2,4-D concentration up to 45.24 µM stimulated direct embryoid cells to be induced from the young leaves L-6 of the spear of oil palm. The same type of direct pearly-white globular cells was reported by 37. Indirect embryogenesis is

more common 38. While direct embryogenesis is more desirable, it is more difficult to achieve as the conditions necessary are more critical than those required to produce embryogenic callus. After 4 subsequent subcultures on the same medium with gradual reduction of auxin concentration, growth of these embryogenic calli and embryoids showed no further development. The mass of tissue maintained this characteristic when cultured in the same medium without changing the medium composition during the time required for this study.
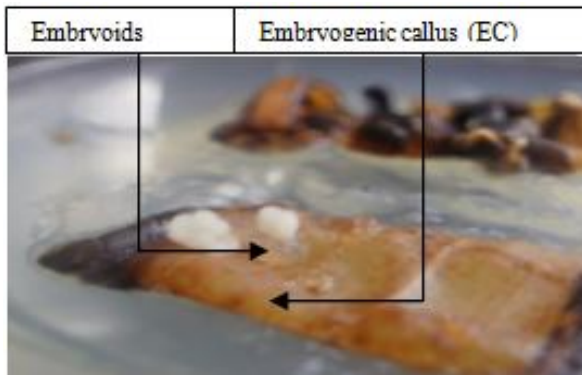
Fig. 6: EC obtained from young leaf n^o -5 of oil palm after 28 weeks of culture on C4 media
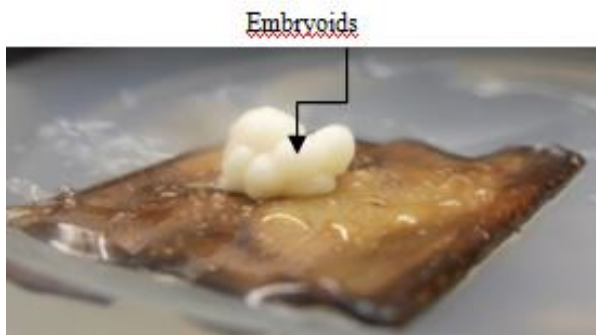
Fig. 7: Direct embryoid cells obtained from young leaf n^o -6 of oil palm in C3 media after 36 weeks of culture.

4. CONCLUSION

The present study successfully describes *in vitro* somatic embryogenesis protocol of young leaf explants of oil palm. MS basal medium containing only NAA (107.41 μM) was the optimal medium for callus induction, while MS basal medium containing the combination of NAA (107.41 μM) and 2,4-D (45.24 μM) was optimal for direct embryoids production of young leaf explants of oil palm var. *Tenera*. No significant difference was observed between the young leaf ranks of oil palm (*Elaeis guineensis* Jacq.) var. *Tenera* for callogenesis. However, the leaf rank L-5 gave the best response for embryogenic callus formation and L-6 for direct embryo production.

5. ACKNOWLEDGEMENTS

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