Journal of Oil Palm Research
DOI: https://doi.org/10.21894/jopr.2023.0064

In Vitro CULTURAL STUDIES FOR CALLOGENESIS AND EMBRYOGENESIS OF OIL PALM (*Elaeis guineensis* Jacq.) USING SEEDLING AND SPEAR LEAF EXPLANTS

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ABSTRACT

In oil palm, hybrids are not homogeneous due to their highly heterogeneous nature. However, the generation of homogeneous material is of prime importance, which can be possible only by using somatic embryogenesis using explants like spear leaf and seedling leaf. Evaluation of callogenesis and embryogenesis of oil palm using seedling and spear leaf is reported here. This is the first report on in vitro *production of callus and somatic embryos using leaves as explants in India. Seedling leaf experiment revealed that callus induction ranged from 3%-4% among the four media combinations used in the study. Somatic embryo induction was observed in modified Murashige and Skoog (MS) containing picloram (37.26 µmol). The minimum time required for callus induction was 29 days whereas the maximum time required for initiation of callus was 5 months and 4 days. The minimum time required for induction of embryogenic callus was noted to be 7 months and 5 days on two different media, whereas, the maximum time was 11 months and 14 days on modified N6 with dicamba (72.39 µmol) (palm no. 377). The present study opens up the prospect of understanding embryogenesis and manipulating* in vitro *culture conditions to achieve plantlet regeneration in oil palm using leaf explant.*

Keywords: oil palm, seedling leaf, somatic embryo, spear leaf.

Received: 29 May 2023; **Accepted:** 30 October 2023; **Published online:** 26 December 2023.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a monocotyledonous, perennial, cross-pollinating, crop belonging to Arecaceae family, cultivated in Africa, Latin America and Southeast Asia. It is now designated as the world's principal vegetable oil crop credited with high productivity and long life spans (Woittiez *et al.,* 2017). In addition to serving as edible oil, its increasing commercial importance in food, soap, and oleo-chemical manufacturing industries; there has been a significant rise in its area under cultivation (19 million hectares) and production of palm oil (81 tonnes) over the last two decades (Murphy *et al.,* 2021). However, to meet the demand for palm oil by ever-increasing population, the multiplication of oil palm plantlets is required on a commercial scale. Multiplication by conventional vegetative propagation of oil palm is not possible because of the presence of a single growing apex, while the seed propagation technique suffers from limitations like a substantial time required for seed germination (1-3 yr) and a very low germination rate (30%) (Martine *et al.,* 2009). The *in vitro* propagation method has evolved as a promising alternative method for the cultivation of oil palm which could address these limitations and has the potential to increase oil production by 20% (Kushairi *et al.,* 2010). Besides this, superior oil quality, reduced palm height (secondary characters), important agronomic characteristics and healthy quality of seeds found in particular elite oil palm genotype could be uniformly expressed in tissue culture-raised plantlets (Paranjothy *et al.,* 1989).

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To date, regeneration of oil palm through somatic embryogenesis has been reported using many explants like immature and mature zygotic embryos (Gomes *et al.*, 2015; 2016; Kanchanapoom and Domyoas, 1999; Monteiro *et al.*, 2017; Teixeira *et al.*, 1995; Thuzar *et al.*, 2011; Wan Nur Syuhada *et al.*, 2016), young plantlets (Scherwinski-Pereira *et al.*, 2010), immature male and female inflorescences (Guedes *et al.*, 2011; Jayanthi *et al.*, 2015; Teixeira *et al.*, 1994), immature leaves (Gomes *et al.*, 2017; Hashim *et al.*, 2018), and seedling leaves (Karun and Sajini, 1994). However, an efficient, universal protocol on the somatic embryogenesis and regeneration system of oil palm is lacking and not repetitive due to several factors, such as genotype, age of the parent tree and explant type (Wecks *et al.*, 2019). Every lab has its protocols for the generation of tissue culture plants in oil palm and hence, the present study aimed at standardising the *in vitro* protocols for callusing and somatic embryogenesis by using the spear and seedling leaves. Furthermore, precise details of the protocols are not available because of the commercial interests of business enterprises involved in micropropagation (Hashim *et al.*, 2018). Since somatic embryogenesis of oil palm is difficult to be conducted owing to recalcitrance, woody and perennial nature of the crop, and is also affected by several factors like medium composition, growth regulators, and explant source, the present study thus endeavours to evaluate the callogenesis and embryogenesis processes by using two different explant types *i.e.*, seedling and spear leaves, for establishing an efficient *in-vitro* regeneration system in oil palm.

MATERIALS AND METHODS

Plant Materials and Culture Initiation

Oil palm seedlings of hybrid *Tenera* (*dura* × *pisifera*) were obtained from the nursery of ICAR-Indian Institute of Oil Palm Research, Pedavegi, Andhra Pradesh, India. The seedlings were first washed with running tap water, and followed by washing with bavistin (1% w/v) for 20 min, and finally washed with 20% (v/v) sodium hypochlorite (HiMedia, India) for 20 min. The outermost leaves were removed destructively, keeping a few interior leaves with the middle column, followed by surface sterilisation with 70% (v/v) ethanol (Merck, India) for 30 s and then thoroughly washed with sterile distilled water for three times, followed by blot drying in a laminar air flow cabinet for 5 min. Subsequently, outer leaf whorls were removed and the leaf lamina and leaf base were taken from the central portion, containing the meristematic region, and later were cut into small pieces (0.5-1.0 cm). To initiate cultures, leaves were inoculated on media, *viz*., modified MS (Murashige and Skoog, 1962), modified N6 (Chu *et al.*, 1975), and modified Y3 (Eeuwens and Blake, 1976), supplemented with growth regulators (*Table 1*). The composition of media used in the present experiment is shown *Table 1*. Cultures were sub-cultured every two months on each respective medium, supplemented with growth regulators for six months, and followed by sub-culturing onto a half-strength respective medium with the same concentration of growth regulators for six months. Cultures were incubated at 26°C to 27°C temperature and ±60% - 65% relative humidity during the entire course of study. The time required for callus induction, somatic embryo formation and percentage of callus induction and somatic embryo formation were noted.

Ortets were selected on the basis of having high yielding capacity of more than 250 kg yr^{-1}, with good bunch parameters (oil to bunch ratio >25%), vegetative growth and healthy condition of the palm, which is free of pests and diseases. The age of selected ortets was 15 years old. The method followed here as per the earlier reports (Hashim *et al.*, 2018). Briefly, all the thorns were removed and fronds were flattened for the collection of the spear leaves which were present within a cylinder of older leaf petioles at the centre of the palm canopy. Subsequently, older fronds and fruit bunches were removed successively to get the cabbage (younger frond petioles) which was cut without disturbing the shoot apex. The cabbage was then placed in a bag and lowered to the ground and the cut ends of cabbage were immediately covered with aluminium foil and brought to the laboratory. The aluminium foil was later removed from the cut ends and the whole cabbage was checked for breakage, freshness and kept in a disease free condition by disinfecting with 70% (v/v) ethanol (Merck, India). Following that, both ends of the cabbage were trimmed and the cabbage was then placed in a laminar airflow followed by frond marking (Monteiro *et al.* 2017). The outermost petiole was later removed by cutting longitudinally and subsequently internal petioles were removed, until the stacks of spear leaves (immature leaflets) were exposed. The immature leaf stacks were cut at 5-6 cm from the distal ends and discarded to avoid any contamination. To initiate culture, sub-stacks of 5-6 leaflets were taken from the stack of leaflets and transferred to another sterile petri plate for explant cutting. These leaflets were cut into strips of about 2 mm and were inoculated on different types of media as mentioned in *Table 3*. The media preparation method and pH conditions were maintained exactly similar as above for the seedling leaf experiment.

The callus percentage was calculated by taking the number of tubes having callus and divided by the total number of tubes in each replication, and expressed in terms of percentage. Likewise, the

TABLE 1. COMPOSITION OF MEDIA USED IN OIL PALM TISSUE CULTURE

	Nutrient composition of media used in the study (mg L^{-1})		
Chemicals	**Modified MS (1962)**	**Modified N6 (Chu *et al.*, 1975)**	**Modified Y3 (Eewens & Blake (1976)**
Macro nutrients			
NH_4Cl	-	-	535
NH_4NO_3	1 650	-	-
$NaH_2PO_4.2H_2O$	-	-	276
KNO_3	1 900	2 830	2 020
$CaCl_2.2H_2O$	440	166	294
$MgSO_4.7H_2O$	370	185	247
KH_2PO_4	-	400	-
KH_2PO_2	170	-	-
$(NH_4)_2SO_4$	-	463	-
KCl	-	-	1 492
Micro nutrients			
H_3BO_3	6.2	1.6	3.1
$MnSO_4.4H_2O$	22.3	4.4	11.2
KI	0.83	0.8	8.3
$ZnSO_4.7H_2O$	8.6	1.5	7.2
$CuSO_4.5H_2O$	0.025	-	0.25
$CoCl_2.6H_2O$	0.025	-	0.24
$Na_2MoO_4.2H_2O$	0.25	-	0.24
NaFeEDTA	37.5	-	-
Na_2EDTA	-	37.2	-
$NaEDTA.2H_2O$	-	-	37.2
$NaMO_4$	-	-	-
$NiCl_2 6H_2O$	-	-	0.024
$FeSO_4.7H_2O$	-	27.8	13.9
Vitamins			
Myo-inositol	100	-	100
Nicotinic acid	0.5	0.5	1.0
Pyridoxine-HCl	0.5	0.5	1.0
Thiamine-HCl	0.1	1.0	2.0
Glycine	200	2	2
Amino acids			
L-Glutamine	100	-	100
L-Arginine	100	-	200
L-Asparagine	100	-	100
Sucrose	30 000	30 000	30 000
Casein hydrolysate	500	500	500
Activated charcoal	3 000	3 000	3 000
Agar	8 000	8 000	8 000

embryogenic percentage was calculated by dividing the number of bottles having embryos and divided by the total number of bottles in each replication. The factorial, randomised complete block design experiments were performed with five replications. The critical difference (CD) and least significant difference (LSD) were calculated to determine if any significant difference existed among the treatments.

RESULTS AND DISCUSSION

The induction of nodular calli from seedling leaf veins were observed after 60 days of inoculation, in four media compositions *i.e.*, modified MS + picloram (37.26 µmol), modified MS + picloram (149.06 µmol), modified Y3 + dicamba (10 µmol) and modified Y3 + dicamba (19.95 µmol), among the 32 media combinations attempted (data not shown). It was observed that the callus induction ranged from 3%-4%, among the four media combinations (*Table 2*). The induced primary calli were white and actively proliferating (*Figure 1*).

There was no morphogenic variability among all the induced calli. It was observed that the primary calli after initiation had induced a single somatic embryo in the modified MS containing picloram (37.26 µmol) (when maintained on its respective medium with growth regulators for 240 days, with every two months of sub-culturing and followed by transferring to respective half-strength medium, supplemented with the same concentration of growth regulators for two months). The other media compositions did not induce any somatic embryos. During these periods, there was no incidence of direct embryogenesis which supported the findings of Karun and Sajini (1994). Moreover, in the present study, it was observed that the induced embryo started to deteriorate in the half strength medium, therefore, it was transferred to a full strength medium, supplemented with the same concentration of growth regulator to retain its embryogenecity, which was similar to Hilae and Te Chato (2005), that suggested a full strength MS, rather than half strength MS for oil palm. Moreover, the role of picloram in the induction of the primary calli and somatic embryo as seen in the present study, also supported the earlier findings which mentioned the suitability of picloram to get embryogenic cultures in different palm species *viz.*, areca palm (Karun *et al.*, 2004), peach palm (Steinmacher *et al.*, 2007), and acai palm.

The spear leaves collected from different palms (*Table 3*), and inoculated on different media supplemented with growth regulators, had resulted into the induction of primary and embryogenic calli. It was observed that palm (P-204), when inoculated on media *viz.*, modified MS + Y3 vitamins, with differing α-napthalene acetic acid (NAA) and dicamba concentrations, had resulted in callus initiation in only 19 days. Whereas, the maximum time required for initiation of primary callus was about 154 days from explants derived from palm (P-377) and inoculated onto modified Y3 supplemented

TABLE 2. *In Vitro* MORPHOGENIC RESPONSE OF SEEDLING LEAF DERIVED CULTURES

Genotype	Media	Primary callus % ± SD
Tenera	Modified MS (1962)+ Picloram (37.26 µmol)	4 ± 0.51[a]
	Modified MS (1962) + Picloram (149.06 µmol)	3.8 ±0.35[b]
	Modified Y3 (Eeuwens and Blake 1976) + Dicamba (10 µmol)	3.84 ±0.29[b]
	Modified Y3 (Eeuwens and Blake 1976) + Dicamba (19.95 µmol)	3.77 ±0.21[b]
	F (cal)	3.799
	CD 5%	0.561

Note: Values with similar alphabet are not significantly different.

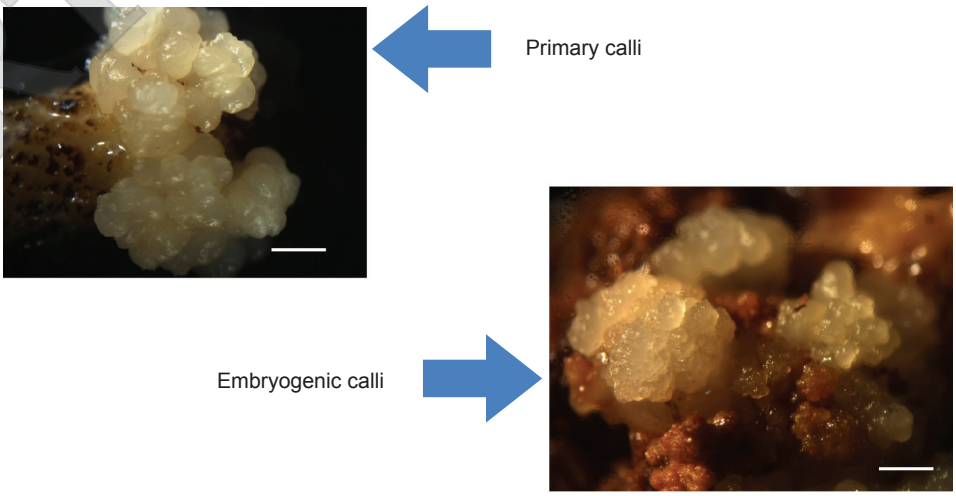

Figure 1. The callus formation in oil palm using seedling leaf tissue (bar = 2 mm).

TABLE 3. *In Vitro* MORPHOGENIC RESPONSE OF SPEAR LEAF DERIVED CULTURES

Genotype	Media	Primary callus % ± SD	No. of months (M) & days (D) required for initiation of primary callus	No. of months (M) & days (D) required for initiation of embryogenic callus
P-419	Modified Y3+ NAA (179.80 µmol)	0.03 ± 0.01^{a}	2 M & 10 D	11 M & 10 D
	Modified Y3 +NAA (119.87 µmol)	0.07 ± 0.02^{b}	2 M & 10 D	9 M
P-204	Modified MS+ Y3 vitamins + NAA (322.23 µmol)	0.03 ± 0.01^{c}	19 D	7 M & 5 D
	Modified MS + Y3 vitamins + Dicamba (72.39 µmol)	0.03 ± 0.01^{c}	19 D	7 M & 5 D
P-377	Modified Y3+NAA_k (534.99 µmol)	0.55 ± 0.07^{c}	5 M & 4 D	-
	Modified N6 + Dicamba(72.39 µmol)	0.28 ± 0.03^{c}	4 M & 5 D	11 M & 14 D
P-251	Modified Y3+Picloram (74.53 µmol)	0.04 ± 0.02^{c}	1 M & 13 D	9 M
	F (cal)	203.001		
	CD 1%	0.057		

Note: Values with similar alphabet are not significantly different.

with NAA (534.99 µmol). The minimum time required for induction of embryogenic callus from the primary callus was 215 days for palm (P-204), and the maximum time was recorded at 344 days for palm (P-377). The maximum percentage of induction of primary callus was 0.55% for palm (P-377).

However, it was observed that there was no induction of somatic embryo from any embryogenic callus, even after maintaining cultures with high auxin and followed by transferring to half concentration of auxin or no auxin, which was contrary to Rival and Parveez (2005). The present study also showed that long exposure of explants to auxin is not required for the initiation of callus in all the palms which is contrary to Hashim *et al.* (2018), where the minimum and maximum time required for callus initiation ranged between 3-12 months. Since the long-term exposure of explants to auxin is not recommended because of the chances of somaclonal variations, the present findings demonstrated the efficiency of medium compositions for early induction of primary callus. In general, oil palm tissue culture suffers from phenolic oxidation, leading to the browning of explants, and therefore activated charcoal was amended in the media (Teixeira *et al.*, 1994; Patcharapisutsin and Kanchanapoom, 1996). The addition of higher concentration of auxin becomes mandatory to achieve callogenesis, since activated charcoal is non-selective (Yusnita and Hapsaro, 2011) and absorbs part of the plant growth regulator. However, the present findings revealed that the concentration of auxins like 2,4-D and picloram, used in the present study, was comparatively less compared to other studies conducted by Gomes *et al.*, (2017), where these were used in the range of 800 µmol and 450 µmol respectively. The similar effect of NAA and dicamba individually in callogenesis has been studied. It was found that a modified Y3 medium supplemented with NAA alone could successfully induce callogenesis. However, some somatic embryos could not be induced, likely due to higher concentrations of NAA used in the present study (179.8 and 119.8 µmol), which might have led to oxidation of cultures and death. Likewise, the role of dicamba in callogenesis and embryogenesis was evaluated in the present study, since dicamba has been used successfully to induce direct and indirect embryogenic callus from tender leaf explants and cultured mature zygotic embryos of oil palm, and has been found suitable for mass propagation of *in vitro* seedlings and mature oil palms. From the present study, it was found that the media *viz.*, modified MS+ Y3 vitamins and modified N6 media containing dicamba (72.39 µmol) could induce primary callus and form embryogenic callus, derived from explants of palms (P-204 & P-377) which is in agreement with Te Chato (1998b), however, without the formation of somatic embryo. One of the factors which was recently found by Aroonluk *et al.*, (2020) indicated that protein phosphorylation mechanism might be playing a major role in the somatic embryogenesis of oil palm. In another set of experiments, a combined effect of auxin and cytokinin for callogenesis was studied. The N6 modified media supplemented with 2,4-D and BAP resulted in the induction of primary callus and embryogenic callus. Although the addition of cytokinin with auxin in oil palm tissue culture is avoided since it causes abnormality, it was still added in the present study to get embryogenic cultures, capable of forming somatic embryos. The identification of genes influencing somatic embryogenesis also helps in screening the germplasm with high potency of embryogenesis (Chan *et al.*, 2020; Zhang *et al.*, 2022). This will

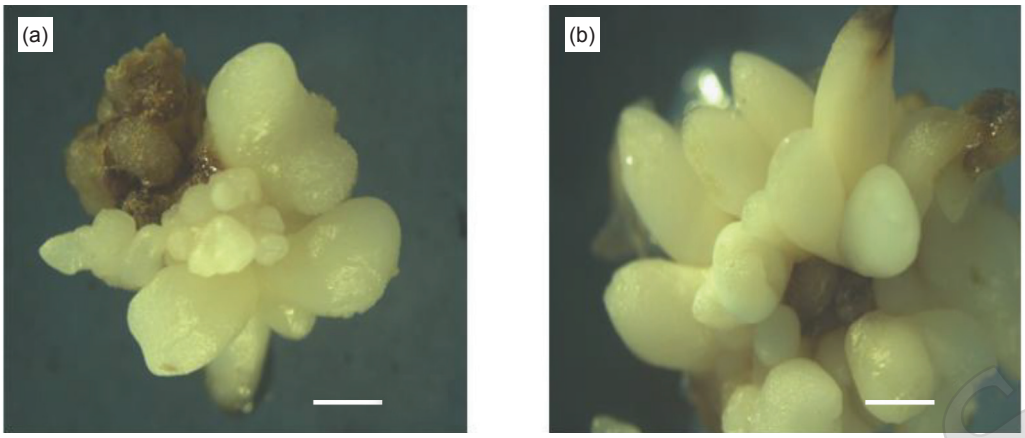

Figure 2. The callus and somatic embryo formation in oil palm by using the spear leaf. (a) Embryo initiation and (b) embryogenic calli with globular and nodular structures (bar=2 mm).

facilitate to identify the palms at an early stage for their use in *in vitro* studies. However, it was observed that the addition of cytokinin along with auxin did not bring any change and ultimately no somatic embryo was induced. The possible explanation of failure of embryogenic cultures to produce somatic embryos in all the palms, can be attributed to its genotypic effect and the presence of varying levels of cytokinin which might have interfered in somatic embryogenesis, irrespective of any media composition used. Moreover, auxin concentration used in the present study might have failed to influence cellular activity to interfere with the endogenous hormones which can trigger gene expression towards re-differentiation for somatic embryo induction.

CONCLUSION

The *in vitro* induction of callogenesis and somatic embryogenesis is a significant step in achieving the *in vitro* organogenesis in oil palm. The present study reported the induction of callus and embryogenic calli from immature leaf and spear leaf explants. The study also reported the minimum time required for initiation of callus, which was 29 days, compared to the maximum time required for initiation of callus, which was about 154 days. Further studies are required to strengthen the *in vitro* regeneration protocols in oil palm, using other explants like immature male inflorescence for generating homogeneous planting material.

ACKNOWLEDGEMENT

The authors would like to acknowledge Director, ICAR-IIOPR, Pedavegi for providing financial support.

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