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Direct Somatic Embryo Formation from Roots of *In Vitro*-Seedlings of Oil Palm (*Elaeis Guineesis* Jacq.)

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Abstract

The induction of somatic embryos (SEs) directly from the roots of oil palm plantlets raised *in vitro*-seedling and origin of somatic embryos. SEs were directly induced from the roots of oil palm plantlets regenerated from immature oil palm fruit (3 months after pollination: 3MAP) and provided by Golden Tenera Company. The plants were cultured on OPCM medium supplemented with 3 % sucrose, 200 mg/l ascorbic acid and 0.5 mg/l NAA. The culture was maintained under light conditions at an intensity of 15 µmol/m^2/s, 14 h photoperiod, 28±1 °C for 2 months. Histological studies revealed that SEs were initiated from epidermal cells and a few parenchymatous cells. Parenchyma cells had a large nucleus, dense cytoplasm with a broad basal area in contact with maternal tissue. Those cells underwent rapid cell division to form proembryonic mass (PEM) and finally developed into SEs through somatic embryogenesis (SE). The highest number of SEs was 1.2 SEs/root and the percentage of SE formation was recorded to be 80. The present study confirms direct SE formation from roots and describes the origin of SE.

Keywords: Oil palm, somatic embryogenesis, *in vitro*-seedling, proembryonic mass (PEM), root

Introduction

Oil palm (*Elaeis guineesis* Jacq.) is a monocotyledon belonging to the family Arecaceae. There are 3 variety types of oil palm which have been classified on the basis of the presence or absence of a shell in the fruit. Dura is a type of fruit with a thick shell. Pisifera has very thin shell or is shell-less while tenera is a hybrid of these 2 types, Dura and Pisifera, and thus, produces fruits with intermediate shells. Naturally, the tenera type produces a large quantity of oil which is obtained from the mesocarp and kernel. Palm oil is used mainly for cooking, the preparation of margarine and also for other applications such as soap, detergent and cosmetics. Oil palm is the most productive oil crop with a yield of up to 5 - 7 tons of palm oil/ha/year [1]. Propagation of oil palm through somatic embryogenesis (SE) is a promising regenerative route, as this morphogenetic pathway may increase the number of regenerated plantlets in comparison with organogenesis [2]. SE is the developmental process by which somatic cells develop into structures that resemble zygotic embryos through an orderly series of characteristic embryological stages without fusion of gametes [3]. This developmental route can be induced by many factors such as explants, culture media, plant growth regulators and culture conditions [4]. Types and concentrations of plant growth regulators (PGRs) in the culture media can greatly affect somatic embryos (SEs) or SE induction of oil palm tissue culture. Regeneration of culture cells is essential for crop improvement through biotechnology. Among the different auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin is the most widely used for oil palm callus induction at a concentration of 1 - 2 mg/l. A much of lower concentration of 2,4-D was applied as the sole plant growth regulator in the regeneration medium to induce regeneration of cultured oil palm cells. Dicamba (3,6-dichloro-o-anisic-acid), another plant growth

regulator and used as a selective systemic herbicide against annual, perennial broad-leaved weeds and bush species [5] is also effective in inducing callus formation. Dicamba has been reported to be an effective auxin for increasing a large number of SEs [6]. Naphthaleneacetic acid (NAA) has also been reported to induce callus formation but a high concentration of 40 mg/l was required [7]. Unfortunately, development of SEs through embryogenesis was not reported. The main effect of NAA was reported to induce the formation of a primary orthogravitropic roots comparable to those developed during *in vitro* germination of oil palm seeds [8].

Another important aspect for adequate morphogenic response is the culture media used [9]. The diverse used of nutritive media has been reported for culturing calli, with positive results in woody plants. [10] used MS (Murashige and Skoog) [11] for callogenesis and subsequent organogenesis of *Alnus sinuate*, as did [12] in the callogenesis and SE based on leaves of the chestnut *Castanea sativa* Mill. Other widely used media with woody plant is the Woody Plant (WPM) [13] which produced the best shoot growth for the micropropagation of the 'Bluecrop' highbush blueberry [14]. OPCM (Oil palm culture media) [15], is a mixture of half strength MS and half strength WPM media and is superior in shoot multiplication and elongation.

Tissue culture techniques have been effectively used to assist the genetic improvement of several crop species. Regeneration of plants from root tissue is not only useful for rapid propagation of clones, but also SEs have been obtained from callus derived from the intact roots of *Prunus incise* × *P. serrula* and horse chestnut (*Aesculus hippocastanum* L.) [16]. Moreover, embryoid structures were also induced on callus nodules developed from the roots of micropropagated cherry rootstock (*Prunus avium* × *P. pseudocerasus*) plantlets [17]. Russell and McCown [18] developed highly competent embryogenic and organogenic systems based on the formation of nodules on the roots of *Populus* shoots rooted *in vitro*. Histological studies describing the origin and different stages of *in vitro* morphogenesis have contributed significantly to the understanding and optimization of various regeneration systems [19]. In this paper, we reported the induction of SEs directly from roots of oil palm plantlets raised *in vitro* and origin of those SEs.

Materials and methods

Plant material

Immature fruits of oil palm breeding material from dura×pisifera (D×P) crosses 3 months after pollination (MAP) were provided by the Golden Tenera Company and used as explants for germination of seedlings. Immature zygotic embryos were aseptically excised and cultured on an MS (Murashige and Skoog) medium supplemented with 2.5 mg/l Dicamba, 3 % sucrose and 200 mg/l ascorbic acid. The medium was solidified with 0.75 % agar, and the pH adjusted to 5.7 before being autoclaved at 1.05 kg/cm^2, 121 °C for 15 min.

Influence of culture media and various concentrations of Dicamba, 2,4 D and NAA on number of SEs and SE formation

Plantlets were cultured on different media (MS, WPM or OPCM) supplemented with various concentrations of Dicamba, 2,4-D or NAA (0.25, 0.50, 0.75 and 1.00 mg/l), 3 % sucrose and 200 mg/l ascorbic acid. The medium was solidified with 0.75 % agar and the pH adjusted to 5.7 before autoclaving at 1.05 kg/cm^2, 121 °C for 15 min. The cultures were placed under light conditions at 15 µmol/m^2/s, 14 h photoperiod at 28±1 °C and sub-cultured after 1 month of incubation. Experiments were carried out by using a completely randomized design (CRD) with 4 replicates (each replicate consisted of 5 plantlets). The percentage of SE formation and the number of SEs developed directly from the *in vitro*-seedling roots were recorded. Means among treatments were separated using Duncan's multiple range test (DMRT).

Histological analysis

For histological observation of somatic embryo derived from the roots, roots with SE samples cultured on OPCM medium supplemented with 0.5 mg/l NAA, 200 mg/l ascorbic acid, 30 g sucrose and

0.7 % agar were fixed by immersing into formalin : acetic acid : alcohol solution (FAA, 1:1:9 v/v) at room temperature for 24 h. The samples were dehydrated through an ethanol series at 30, 50, 70, 85, 95 and 100 %, respectively at 2 h for each concentration. The samples were embedded in Paraffin wax at 60 °C, sectioned with rotary microtome at 8 µm and fixed on glass slides. The sections were de-waxed in xylene for 10 min, stained with Delafield's Hematoxylin and Johansen's Safranin solution for 15 min [20] and observed under a light microscope (Olympus CKX 41).

Results and discussion

Effect of culture medium on number of SEs and SE formation

Different PGRs gave a different response on the number of SEs and SE formation. Plantlets cultured on a MS medium without a plant growth regulator, did not induce SE formation (**Figure 1A**). Whereas, a MS medium supplemented with 0.5 mg/l NAA resulted in the highest number of SEs at 0.45 SEs/root and percentage of SE formation at 35 (**Table 1**). SEs derived from the roots directly were creamy to green color (**Figure 1B**).

NAA at concentrations of 0.50 mg/l with WPM gave the highest number of SEs at 0.7 SEs/root, followed by 2,4-D at 0.25 mg/l which gave the number of SEs at 0.6 SEs/root. However, the percentage of SE formation obtained from 0.5 mg/l NAA and 0.25 mg/l 2,4-D containing media gave equal results at 50 (**Table 1**). These SEs were yellow to green in color (**Figure 1D**).

SEs was successfully germinated into seedlings, shoots with roots. The best results were for SEs initiated on an OPCM medium supplemented with 0.5 mg/l NAA after 2 months of culture. The highest number of SEs at 1.20 SEs/root and percentage of SE formation at 80 was obtained in the OPCM medium (**Table 1**). SEs developed on this medium were yellow to green in color and germinated directly into shoots and roots, either singly or in group (**Figure 1C**).

Plant regeneration under *in vitro* conditions depends on various factors, such as, types of explant, basal medium, growth regulators, genotypes and culture conditions. Auxin, particularly 2,4-D or NAA are known to trigger the stimulation of pre-embryogenic determined cells to undergo cell division and then expression of embryogenesis in many plant species [21]. The efficient regeneration of SE from different plant species depends strongly on the medium composition. The OPCM medium is frequently used to induce SE from culturing various explants of oil palm. Although the OPCM medium has not been reported for use as a culture medium for oil palm tissue culture before, we found that the OPCM medium was the most suitable for this purpose in the present study. In contrast, MS and WPM medium gave only a low frequency of SE induction. The macro- and microelements in the OPCM medium were very different to the MS and WPM media. The OPCM medium contains a higher concentration of Cl^- than the WPM medium due to the presence of $CuCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$. Although Cl^- promotes root formation in the same manner as natural auxins, it may also play an important role in the growth and development of SEs in oil palm. The presence of NH_4NO_3 is thought to prevent the division of tissue in many woody plant species, such as poplar [22]. In the present study, we found that a higher induction of SEs in OPCM medium might be due to an appropriate ratio of NH_4^+ to NO_3^-. The ratio of these chemicals was 4.1:3.8 with a higher concentration of NH_4^+ than that present in MS and WPM (**Table 2**). We believe this to indicate a nitrogen deficiency. In the order to increase the nitrogen concentration in the medium, we initially planned to use MS, which contains 4 times as much nitrogen a WPM (1,650 and 400 mg/l) (**Table 2**) and is widely used for plant tissue culture. However, MS is reported to be less effective than WPM in the micropropagation of Bluecrop [23]. Therefore, we used OPCM as an intermediate nitrogen medium between MS and WPM. In the present culture, the average number of SEs and SE formation on MS medium supplemented with various concentrations of Dicamba was poorer than that on the other media. OPCM was superior in SE induction and SE formation although plant growth regulator effects were still observed (**Table 1**). With respect to 'Bluecrop', the present results obtained for shoot multiplication and elongation on MS and WPM were opposite to those reported by [23], who found WPM to be superior. Another explanation may be a deficiency of microelements, since WPM contains neither cobalt nor iodine. MS often produces hyperhydric shoots because it is rich in the ammonium ion [24]. Thus, to avoid generating hyperhydric shoots OPCM is preferable to MS.

In the present study, SEs were obtained from *in vitro*-roots of plantlets on MS, WPM or OPCM medium supplemented with different concentrations of Dicamba, 2,4-D or NAA. The OPCM medium supplemented with different concentrations of NAA showed a better response in term of SE formation and number of SEs than those of MS or WPM medium enriched with Dicamba or 2,4-D. 2,4-D has previously been the most commonly used auxin for the induction of SE in the tissue culture of oil palm [25]. But in this study, we found that NAA showed more potential to induce SE than 2,4-D. NAA has also been reported to promote plantlet elongation with significant differences in comparison with Dicamba and 2,4-D [26]. However, other kinds of auxin did not promote plantlet growth. In many cases, embryogenesis is reported to occur in many stages, such as the induction of cells which undergo division to form embryogenic mass or proembryonic tissue in the presence of high concentrations of auxin and the development of embryogenic mass into SEs in the absence, or presence of a low concentration of auxin [27]. Vankatachalam *et al*. [28] observed a higher frequency of SEs germination in groundnuts in the present of 8.88 µM BA and 0.26 µM NAA. Lehminger-Mertens and Jacobse [29] have reported germination of SEs derived from protoplast of peas on a MS medium supplemented with 1.5 - 2.9 µM GA_3.

Table 1 Effects of culture media supplemented with different concentrations of Dicamba, 2,4-D or NAA on the number of SEs and percentage of SE formation after culturing for 2 months.

Plant growth regulators	Concentrations (mg/l)	Avg. no. of SEs±SD			SE formation (%)		
		MS	WPM	OPCM	MS	WPM	OPCM
Dicamba	0	0.00±0.00	0.00±0.00	0.00±0.00	0	0	0
	0.25	0.20±0.00	0.35±0.05	0.30±0.06	20	25	25
	0.50	0.00±0.00	0.20±0.00	0.25±0.05	0	20	20
	0.75	0.00±0.00	0.00±0.00	0.00±0.00	0	0	0
	1.00	0.20±0.00	0.00±0.00	0.25±0.05	0	0	20
2,4-D	0	0.00±0.00	0.00±0.00	0.05±0.05	0	0	5
	0.25	0.00±0.00	0.60±0.00	0.50±0.06	0	50	30
	0.50	0.20±0.00	0.40±0.00	0.25±0.05	20	20	20
	0.75	0.25±0.05	0.00±0.00	0.05±0.05	25	0	5
	1.00	0.20±0.00	0.00±0.00	0.15±0.05	20	0	15
NAA	0	0.00±0.00	0.00±0.00	0.00±0.00	0	0	0
	0.25	0.00±0.00	0.40±0.00	0.30±0.06	0	40	25
	0.50	0.45±0.05	0.70±0.06	1.20±0.08	35	50	80
	0.75	0.15±0.00	0.20±0.00	0.90±0.05	15	20	55
	1.00	0.35±0.05	0.20±0.05	0.70±0.05	30	20	50
F-test			ns				ns
C.V. (%)			38.27				25.12

ns: not significant difference

Table 2 Components of different culture media used for culturing of oil palm plantlets.

Elements	Concentration (mg/l)		
	MS	**WPM**	**OPCM**
Macronutrients			
NH_4NO_3	1,650.000	400.000	1,025.000
KNO_3	1,900.000	-	950.000
KH_2PO_4	170.000	170.000	170.000
$CaCl_2.2H_2O$	440.000	96.000	268.000
$MgSO_4.7H_2O$	370.000	-	185.000
Micronutrients			
KI	0.830	-	0.415
K_2SO_4	-	990.000	495.000
H_3BO_3	6.200	6.200	6.200
$MnSO_4.H_2O$	16.900	16.900	16.900
$ZnSO_4.7H_2O$	10.600	8.600	9.600
$CuSO_4.5H_2O$	0.025	6.250	3.138
$Na_2MoO_4.2H_2O$	0.250	0.250	0.250
$CuCl_2.6H_2O$	0.025	-	0.0125
$FeSO_4.7H_2O$	27.800	27.800	27.800
Na_2EDTA	37.300	37.300	37.300
Organics			
Myo-inositol	100.000	100.000	100.000
Nicotinic acid	0.500	0.500	0.500
Pyridoxine HCl	0.500	0.500	0.500
Thaiamine HCl	0.100	0.100	0.550
Glycine	2.000	2.000	2.000

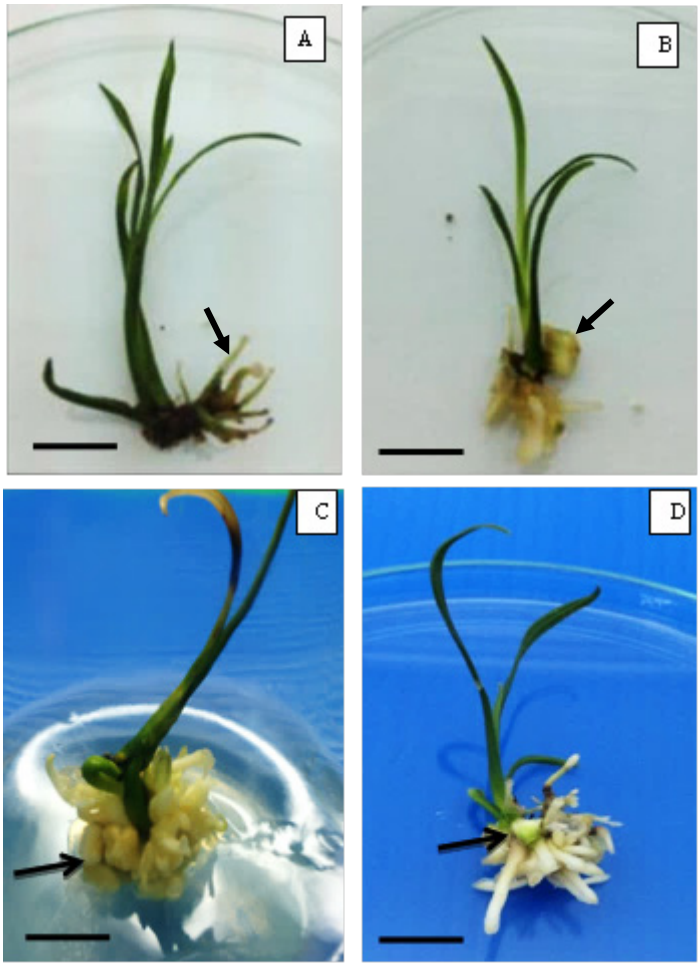

Figure 1 Direct induction of SE from root of oil palm plantlet after culture for 2 months. (A) Plantlet cultured on a MS medium without growth regulators, showing normal roots (arrow). (B) Plantlet cultured on a MS medium supplemented with 0.5 mg/l NAA, showing a somatic embryo (arrow). (C) Plantlet cultured on an OPCM medium supplemented with 0.5 mg/l NAA, showing a somatic embryo (arrow). (D) Plantlet cultured on a WPM medium supplemented with 1.0 mg/l NAA, showing a somatic embryo (arrow) (bar = 1 cm).

Histological

Histological analysis of yellow to green SEs differentiated directly on *in vitro*-plantlet roots either singly or in groups after 1 - 2 months of culture revealed that most SEs developed in the proximal region of a single root. Subsequent development of SEs was not synchronous. SEs appeared to differentiate from epidermal cells and a few parenchymatous cells. Parenchyma cells had a large nucleus, dense cytoplasm with a broad basal area in contact with maternal tissue. They underwent rapid cell division to form proembryonic mass (PEM) and finally developed into SEs through SE (**Figure 2C**). The longitudinal root tip section from the PGR-free MS medium showed a normal structure with a root cap, calyptrogens (actively dividing root cap cells), quiescent center and procambium (**Figure 1A**). The longitudinal section of the SE showed a parenchymatous linkage between the root and SE. All SEs had a vascular connection from shoot apex to root zone or radicle (**Figure 2C**). However, some roots were not initiated to SEs

(**Figure 2B**). Histological analysis reveals cells with embryogenic characteristics, such as small cells with small vacuoles, large nuclei and dense cytoplasm. Such cells are usually found as niches of sub epidermal and epidermal tissue. In coconut SE, sub epidermal embryogenic cells also had a dense cytoplasm [30]. Additionally, oil palm SEs appeared with a broad basal area in contact with maternal tissue, and without vascular connection to the maternal tissue, and histological analyses showed no co-ordination with cell division during the initial development. All these characteristics suggest a single cell origin of the SEs [31], however plantlets have been regenerated mostly from SEs with a multicellular origin [32].

This could be because the OPCM and WPM have lower concentrations of macronutrients than the MS media, in particular lower concentrations of Ca and N (**Table 1**). The composition of the culture media has an important influence on the morphogenic response, as was demonstrated in the numerous investigations conducted by [33] which showed that the morphogenic response of a determined tissue is associated with the mineral nutrition that is applied. It is even possible to eliminate growth regulators by modifying the mineral composition of the culture medium [34].

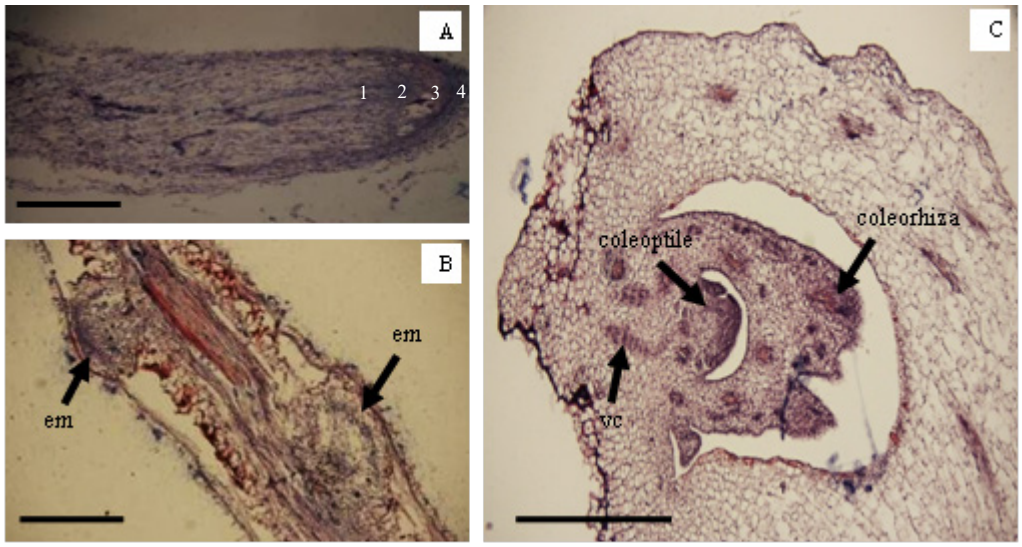

Figure 2 Histological studies of roots and SE of oil palm after 2 months of culture. (A) Longitudinal sections of the root from PGR-free MS medium without SEs (bar = 501.53 µm) showing a normal root tip with 1; procambium, 2; quiescent center, 3; live root cap cells, 4; root cap. (B) Longitudinal sections of the root bearing SEs (bar = 501.53 µm). (C) Longitudinal sections of a somatic embryo (bar = 800 µm). vc = vascular cambium; em = embryonic mass.

Conclusions

We report high frequency direct SE of oil palm by culturing plantlets on an OPCM medium supplemented with 0.5 mg/l NAA. Roots of *vitro*-plantlets gave the highest number of SEs at 1.2 SEs and percentage of SE formation at 80. Histological studies revealed that SE appeared to differentiate from epidermal cells and few parenchymatous cells. Parenchyma cells appeared with a broad basal area in contact with maternal tissue, and without vascular connection to the maternal tissue. This morphogenetic pathway allowed the development of a protocol suitable for direct somatic embryo formation from roots of *in vitro*-seedlings of oil palm (*Elaeis Guineesis* Jacq.).

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