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Evaluation of effect of age on callus initiation rate in oil palm (*Elaeis guineensis* Jacq.) Types

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Abstract

The need to overcome the constraints in oil palm propagation with seeds resulted in somatic embryogenesis (plantlets production). So far, this has yielded limited success. Consequently, effort to improve the technique, calls for evaluation of the effect of palm age, one of the in vitro cultural factors, on callus production rate. A factorial experiment (2x2x3x3x 4) in completely randomised design, with eighteen replicates was conducted. Immature terminal leaves harvested from 8, 10, 12 and 14 year old Dura, Tenera and Pisifera oil palm types were analyzed for mineral content and cultured in Murashige and Skoog, modified MS and Eeuwens media. Each was supplemented with 160mgl^{-1} Naphthalene acetic acid (NAA) or 22 mgl^{-1} 2,4-Dichlorophenoxy acetic acid (2,4-D). Explants were incubated in uninterrupted light or darkness. Calli initiation from leaf explants were recorded and expressed in percentages. Data were subjected to ANOVA at P= 0.05. Callus initiation occurred in all oil palm types across the palm ages irrespective of light regime. Callusing decreased significantly with age from 10.5% (8 years) to 4.8% (14 years). The younger palms which exhibited higher rates of calli initiation than older palms also contained higher levels of essential minerals. Somatic embryogenesis has the advantage of non-destruction of parent plants and production of true-to-type multiple offspring when exploited. This would involve young (8 years) palms containing required amounts of essential elements and Eeuwens medium supplemented with optimal concentration of growth regulators (NAA or 2,4-D).

Keywords: Callus initiation, limited success, palm age, overcome, significantly.

Introduction

The establishment of commercial cultivated area of selected clones can result in benefits such as uniformity of harvest, simple organization practices and optimization of the production of oil in addition to rapid multiplication of the superior genotypes[1].

For the clonal proliferation of oil palm, the somatic embryogenesis has been the most utilized technique[2,3]. This has been applied on different explants, such as juvenile leaves of young plants, juvenile inflorescences, and zygotic embryos[4,5]. In Agriculture and plant science, the practical use of *in vitro* method of propagation for plantlet regeneration has been for a long time but it assumed new levels of emphases among scientists in diverse field of studies in recent years. The success of the technology is affected by different factors including age[6,7] and the system proceeds in stages. For example, somatic embryogenesis is separated into four (call genesis, embryogenesis, shoot development and rooting). In many plants, morphogenesis is characterized by extended delays resulting from the inefficiencies of some of the stages of the process and the polyphenols produced by the explants in culture. This is reported to affect the success of regeneration of plants in a manner that is not yet clearly understood. Therefore, protocol optimisation requires painstaking effort particularly at the callus initiation[8,9]. In the oil palm, somatic embryogenesis technology which had been reported recurrently is known to produce low average callus initiation rate[8,10,11]. The need to improve the callus production rate of the culture system calls for investigation of the effect of the cultural factors necessary for success. Consequently, there search was set-to evaluate the effect of palms age on callus initiation rate in oil palm types.

Materials and methods

This research was conducted in the Plant Physiology and Tissue Culture Division, Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Nigeria.

Plant material used: The immature leaf explants used for this study were obtained from the Seed Production Division (SPD), NIFOR, Benin City. Explants were harvested from Dura, Tenera

and Pisifera palms at four different age groups (8, 10, 12 and 14 years).

Culture and media preparation: The basal media used were Murashige and Skoog[12], modified MS and Eeuwens[13]. These were used together with organic supplements (myo-inositol, $0.1gl^{-1}$; Thiamine-HCl, $0.005gl^{-1}$; Pyridoxine-HCl, $0.005gl^{-1}$; nicotinic acid, $0.005gl^{-1}$ and adenine sulphate, $0.004gl^{-1}$). The Eeuwens medium was augmented with 45 gl^{-1} sucrose, while the MS media were augmented with 30 gl^{-1} sucrose. Each of the three culture media, Murashige and Skoog (MS)[12], modified MS and Eeuwens[13] used in the study were in two sets. One set was cultured in media supplemented with 160mg l^{-1}. NAA and the other set was supplemented with 22 mg l^{-1} 2,4-D. There were thus a total of 144 treatment combinations (Table-1). Each of the treatments was replicated eighteen times. The pH of the culture media was adjusted to 5.7 ± 0.1 using a pH meter for measurement and 0.1N hydrochloric acid (HCl) as well as potassium hydroxide (KOH) prior to autoclaving[2]. 7g agar and 2.5g activated charcoal were added to 1litre solution each of the medium. The media and agar were then heated to 60^oC for 35 minutes in the autoclave to melt the agar. The agar mixed with the media components and solidified on cooling to produce semi-solid state. The compounded solutions, before cooling down to ambient temperature were dispensed as 20ml aliquots into test tubes. These were sterilised in an autoclave at steam pressure of 15 psi at 120^oC for 15 minutes.

Explant sterilization and inoculation: With the use of running tap water the explants were thoroughly washed for 10 mins, before taken to chamber room, for proper sterilization. The explants were cut into fragments of about 5cm in length with the aid of a scalpel, placed in twelve different clean 500ml capacity beakers each having a lid for cover and labeled according to type and age. 2.6% sodiumhypochlorite (NaOCl) solution, 3.6 litres in volume was prepared and 8 drops of 'Teepol' (Detergent solution) was added in order to improve the penetration of the tissues of the explants by the sterilant. The NaOCl solution was shared equally into the twelve beakers each of which contained one explants representing any one of the oil palm types of Dura, Tenera and Pisifera from one of the four age groups were fully submerged in the solution. The explants were allowed to soak in the NaOCl solution for 15 minutes. The beakers containing the explants were regularly agitated to ensure complete wetting of the explants. The solution was drained off and the explants in the beaker were rinsed three times with distilled water.

The beakers containing the sterilised explants were then covered and left inside the cabinet before inoculation. A leaf slice of about 1.5 to 2.0cm long portion was inoculated on the culture media in each test tube. The tubes represented the experimental units.

Half of the experimental units were incubated at 27 ± 1^oC under uninterrupted light with intensity of 1000 lux which was provided by warm white fluorescent lamps as recommended by Narayanaswamy[14]. The other half (set) of the experimental units were incubated at same temperature, but in uninterrupted darkness. The factorial experiment (2x2x3x3x4) was laid out in the incubation chamber in a completely randomised design.

Monthly subculture: Cultures were transferred every four weeks from the old culture media to new media of the same composition under aseptic conditions. The proportion or percentage of explants which initiated calliper treatment was recorded.

$$\text{Thus, callus initiation (\%)} = \frac{\text{No. of explants forming callus}}{\text{Total no. of explants units cultured}} \text{ x } 100$$

The cultured explants were inspected daily while records of observation were taken every two weeks for a period of sixteen weeks. Photographs of the initiated callus and other responses were taken.

Extraction of leave sample: Leaf samples were taken from selected oil palm stands (in the seed garden) as the source of explants. A sample of leaf No. 17 (counted from the first fully opened frond in the centre of the crown frond being No. 1) was taken from each of Dura, Tenera and Pisifera oil palm types of 8, 10, 12 and 14 years of age, in line with the recommendations by Ochs and Olivin[15].

A five-gram sample of each leaf was taken. In order to obtain a constant weight of samples, they were oven-dried at 70^oC forthree days. Dried samples were milled with a milling machine and the milled samples were analysed for per cent content of Nitrogen, phosphorus potassium, magnesium calcium, sodium and sulphur using standard procedures

Statistical analysis of data: The data collected were subjected to analysis of variance (ANOVA) at P = 0.05 and the means separated using LSD. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 14.

Results and discussion

Leaf explants callogenesis initiation in oil palms of different ages cultured in media supplemented with determined optimum concentrations of NAA and 2,4-D.

Under uninterrupted light, callus inoculation rates decreased significantly with age of palm (Table-2). Averaged over oil palm types and culture media callus initiation rate was lower with 2,4-D than NAA at 8,10, and 12 years of palm age. At 14 years palm age rate was higher with 2,4-D. Under uninterrupted darkness callus initiation also declined with age as was the trend under uninterrupted light. Callus initiation rates in darkness when averaged over oil palm types and culture media were also lower with 2,4-D than NAA at 8 and 10 years palm age but at 12 and 14 years the rates were similar or higher with 2,4-D.

Thus in both light regimes all the oil palm types initiated call us at all palm ages. Averaged over oil palm types and culture media, using data from Table-2, the callus initiation values of leaf explants were 10.5, 8.7, 7.0 and 4.9% for 8, 10, 12 and 14 years old oil palm, respectively; further confirming the decline of callus initiation rate with age in either light or darkness. There was also significant difference in callus initiation in light and darkness with 7.3% and 8.5% respectively, average over palm ages, oil palm types, culture media and plant growth regulators.

It is noteworthy that there was only one significant interaction among the various factors and this was between the palm ages and oil palm types for callus initiation rates. This indicates differences among the oil palm types in their pattern of callus initiation rates with age. Thus, while the highest callus initiation rates with Pisifera were consistently with 8 years old palms (Table-3) higher or similar values with 8 years old palms were also recorded with either 10 or 12 years old of Dura and Tenera. The lowest rates among all types were with 14 years old palms. It may be concluded that 8 to 12 years old palmss would be the most responsive to culturing.

The mineral element contents of fresh leaves of oil palm types of different ages, used as explants are shown in Table 4. In general mineral element content declined with age although there were some exceptions. The critical levels of the constituents are as provided by Ochs and Olivin[15], Foster and Goh[16] and Uexkull[17].

Table-1: Factorial combinations of Treatments in Experiment.

Light regime	Culture media	Plant growth regulator	8			10			12			14		
			[1]Du	[2]Te	[3]Pi	Du	Te	Pi	Du	Te	Pi	Du	Te	Pi
Uninterrupted light	MS	NAA	T_1	T_3	T_5	T_7	T_9	T_{11}	T_{13}	T_{15}	T_{17}	T_{19}	T_{21}	T_{23}
		2,4-D	T_2	T_4	T_6	T_8	T_{10}	T_{12}	T_{14}	T_{16}	T_{18}	T_{20}	T_{22}	T_{24}
	Mod.MS	NAA	T_{25}	T_{27}	T_{29}	T_{31}	T_{33}	T_{35}	T_{37}	T_{39}	T_{41}	T_{43}	T_{45}	T_{47}
		2,4-D	T_{26}	T_{28}	T_{30}	T_{32}	T_{34}	T_{36}	T_{38}	T_{40}	T_{42}	T_{44}	T_{46}	T_{48}
	Eeu	NAA	T_{49}	T_{51}	T_{53}	T_{55}	T_{57}	T_{59}	T_{61}	T_{63}	T_{65}	T_{67}	T_{69}	T_{71}
		2,4-D	T_{50}	T_{52}	T_{54}	T_{56}	T_{58}	T_{60}	T_{62}	T_{64}	T_{66}	T_{68}	T_{70}	T_{72}
Uninterrupted darkness	MS	NAA	T_{73}	T_{75}	T_{77}	T_{79}	T_{81}	T_{83}	T_{85}	T_{87}	T_{89}	T_{91}	T_{93}	T_{95}
		2,4-D	T_{74}	T_{76}	T_{78}	T_{80}	T_{82}	T_{84}	T_{86}	T_{88}	T_{90}	T_{92}	T_{94}	T_{96}
	Mod MS	NAA	T_{97}	T_{99}	T_{101}	T_{103}	T_{105}	T_{107}	T_{109}	T_{111}	T_{113}	T_{115}	T_{117}	T_{119}
		2,4-D	T_{98}	T_{100}	T_{102}	T_{104}	T_{106}	T_{108}	T_{110}	T_{112}	T_{114}	T_{116}	T_{118}	T_{120}
	Ee	NAA	T_{121}	T_{123}	T_{125}	T_{127}	T_{129}	T_{131}	T_{133}	T_{135}	T_{137}	T_{139}	T_{141}	T_{143}
		2,4,-D	T_{122}	T_{124}	T_{126}	T_{128}	T_{130}	T_{132}	T_{134}	T_{136}	T_{138}	T_{140}	T_{142}	T_{144}

Key: [1]Du, Dura; [2]Te, Tenera; [3]Pi, Pisifera.$T_1 – T_{144}$, Treatment combinations.

Table-2: Effects of oil palm types and age on leaf explants callus initiation rates indifferent light regimes averaged over culture media.

Plant growth regulator	Oil palm types	Uninterrupted light				Mean	Uninterrupted darkness				Mean
		Palm age (yr)					Palm age (yr)				
		8	10	12	14		8	10	12	14	
----------%----------											

$^{+}$(160 mgl^{-1} NAA)	Dura	12.2	11.1	8.5	5.9	9.4	13.7	14.1	10.0	7.1	11.2
	Tenera	11.1	11.5	6.7	3.0	7.2	14.4	11.1	5.5	2.7	8.4
	Pisifera	9.3	7.4	5.9	3.7	6.6	12.9	7.8	3.3	5.6	7.4
$^{+}$(22 mgl^{-1} 2,4-D)	Dura	8.5	8.9	9.6	7.4	8.6	10.7	9.3	8.9	7.1	9.0
	Tenera	8.2	9.3	6.7	5.6	7.4	10.4	9.3	9.3	6.7	8.9
	Pisifera	5.9	4.8	3.7	2.6	4.3	5.9	4.4	3.7	3.3	4.4
	LSD(0.05)	3.1	2.9	3.3	2.9	1.8	4.7	4.5	ns	3.2	2.3

Key: LSD (0.05): at P= 0.05 for comparing means in columns, values in brackets are the determined optimal plant growth regulator concentrations.

Table-3: Leaf explants callus initiation in different ages averaged over oil palm types, culture media and light regimes.

Palm age	Leaf explants initiation of callus (%)		Mean
	Uninterrupted light	Uninterrupted darkness	
Years	----------%----------		
8	9.6	11.4	10.5
10	8.1	9.3	8.7
12	6.7	7.2	7.0
14	4.7	5.0	4.9
LSD (0.05)	1.3	1.9	1.1
Mean	7.3	8.5	

Key: +mean values in the row are significantly different (P= 0.05).

Table-4: Essential mineral element contents (%) of leaves of oil palm types at different ages[15-17].

Oil palm type	Palm age	Minerals						
		N	P	K	Mg	Ca	Na	S
	---yr---	----------%----------						

Dura	8	1.05	0.23	1.68	0.18	0.34	0.14	1.74×10^{-2}
	10	0.92	0.23	2.63	0.15	0.31	0.14	2.01×10^{-2}
	12	0.60	0.19	2.02	0.14	0.29	0.14	1.68×10^{-2}
	14	0.49	0.16	2.13	0.14	0.27	0.09	1.77×10^{-2}
Tenera	8	0.98	0.27	1.82	0.20	0.38	0.14	1.84×10^{-2}
	10	0.85	0.23	1.52	0.21	0.43	0.09	1.44×10^{-2}
	12	0.78	0.21	1.16	0.22	0.37	0.09	1.79×10^{-2}
	14	0.45	0.21	1.21	0.23	0.41	0.14	1.80×10^{-2}
Pisifera	8	0.98	0.23	2.69	0.04	0.51	0.09	1.53×10^{-2}
	10	0.82	0.23	1.63	0.15	0.41	0.19	1.77×10^{-2}
	12	0.78	0.22	0.96	0.20	0.38	0.14	2.15×10^{-2}
	14	0.53	0.22	0.32	0.23	0.31	0.14	1.69×10^{-2}
		*2.30	*0.15	*0.8 -10	*0.24	*0.60	*0.10-0.20	$*27 \times 10^{-2}$

Key: *, Oil palm critical levels.

Discussion: The results showed that age of oil palm from which explants were taken affected callus initiation. The younger the age of the explants sources the greater the chances of callus initiation. This was the situation in the present study with three oil palm types whose age ranged from 8 to 14 years. This agrees with the report of Hartmann *et al*.[6] that success in micro propagation of woody plant species is, to a large extent, a function of the juvenility of the source plants. This implied that young plants are easier to micropropagate than more mature plants of the same species. Osifo[7] reported that the young leaflet explants of *solanumbrevidens* were more responsive to *in vitro* stimuli for callus and Shoot formation than the old leaflet tissues while Pierik *et al* reported that tissues and organs of juvenile plants are much more responsive than tissues or organs of adult plants in processes such as cell division and formation of adventitious organs[18]. Although there were differences in the age of the oil palm trees i.e. palms from which explants were harvested for the present study, the fronds from which the explants were obtained was frond 'No. 1' in the newly opened leaf cabbage. These fronds were the youngest. Thus the leaf explants from the palms of various ages were fresh and relatively of the same level of succulence in spite of the fact that the age of the palm significantly affected callus initiation as the results show. The need to explain the situation led to the chemical analysis to determine the content of essential nutrient elements in the leaf explants especially those elements which are fundamental to the growth and development of promeristematic cells and ultimately plantlets/plants organs. Thus the results of the chemical analysis of the leaf explants revealed that nitrogen, phosphorus, potassium and calcium content decreased as palm age increased. Therefore the 8 years old palms were richest in major nutritive elements with the lowest content in 14 years old palms. As noted above, the number of leaf explants portions which formed callus also followed the same trend with the youngest palms producing the most calli and the oldest producing the least. It can therefore be concluded that the age of palms affects the quantity of nutritive elements present in newly opened leaves/fronds from which leaf explants are selected for culture. The younger the palm, the richer the central frond i.e. explants in essential nutrient elements. Consequently, the higher the explants nutrient content the higher the number of calli that can be generated by the explants and the greater the number of generated calli with potential to develop into somatic embryos and ultimately, plantlets.

Complete sequence of events at the apices in somatic regeneration involves division, enlargement, differentiation and maturation of cells leading to origin of new organs[19]. The growth of cells of the promeristem involves the synthesis of plant substances, especially protein and nucleic acids, within the cells and the needed raw materials such as sugars, amino acids, phosphates, potassium and other minerals must be sufficiently available[13]. Consequently, it is essentially the differences in these raw materials among leaf explants from the 8, 10, 12 and 14 years old palms that can explain the difference in callus initiation which declined with increasing palm age in all three oil palm types.

Conclusion

Callus initiation declined with age of palms. Averaged over oil palm types, culture media and plant growth regulators, callus initiation rates in light were 9.6%, 8.1%, 6.7% and 4.7% for 8, 10, 12 and 14 years old palms, respectively. In darkness the rates were 11.4%, 9.3%, 7.2% and 5.0%, respectively. The younger palms which exhibited higher rates of callus initiation than older palms also contained higher levels of essential minerals than older palms.

Figure-1: Freshly inoculated immature oil palm leaf explants in nutrient medium.

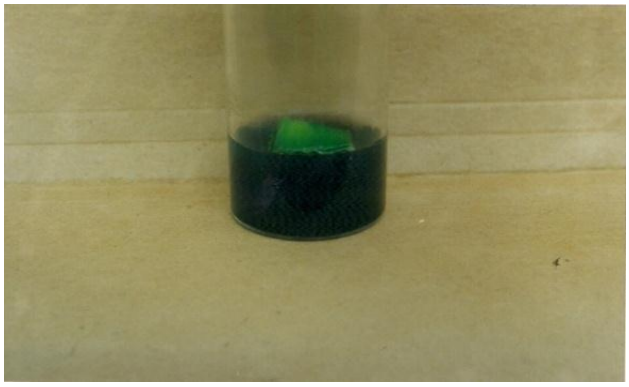

Figure-2: Oil palm leaf explants turned green after four week incubation under uninterrupted light.

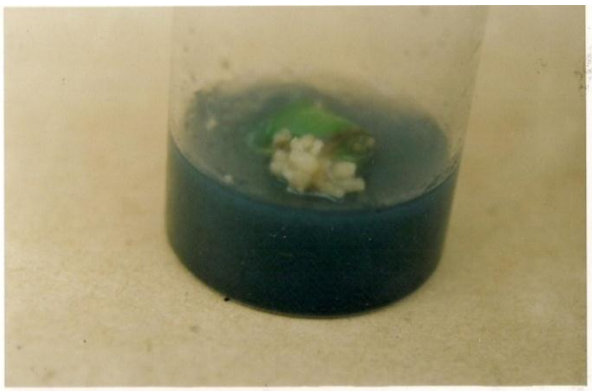

Figure-3: Oil palm leaf explants initiated callus after four weeks incubation under uninterrupted light.

Figure-4: Oil palm leaf explants turned yellowish when incubated under uninterrupted darkness for four weeks.

Figure-5: Chlorotic oil palm leaf explants initiated callus in four to five weeks of incubation under uninterrupted darkness.

Figure-6: Embryonic calli (white coloured) initiated from oil palm leaf explants (green) in culture.

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