Plant Cell Biotechnology and Molecular Biology 20(7&8):314-323; 2019 ISSN: 0972-2025

EFFECT OF ACTIVATED CHARCOAL, CULTURE MEDIA AND PLANT GROWTH REGULATORS ON *In vitro* GERMINATION AND DEVELOPMENT OF ELITE DURA OIL PALM *(Elaeis guineensis Jacq.)* ZYGOTIC EMBRYOS

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Received: 15 April 2019
Accepted: 29 June 2019
Published: 04 July 2019

Original Research Article

ABSTRACT

Innumerable usage and huge economic importance of oil palm necessitates the wider spectrum of genetic variability, but its narrow genetic base makes major constraint in achieving the genetic variance which mandates the conservation of promising and elite material in field gene bank, which is resources demanding. In this context tissue culture has become an only promising alternative, and among several explant sources, zygotic embryo could achieve a repeatable results in *in vitro* germination. Hence in this study the effect of culture media (CM), activated charcoal (AC) and plant growth regulators (PGR) were assessed with 12 treatments on initial differentiation and further development of zygotic embryos. Elite Dura ZEs were cultured on MS,N6&Y3 media, with or without AC (2g L^{-1}) and with or without PGR (NAA, BA& GA3-0.2mg L^{-1}). AC has a significant effect on initial differential stages of ZEs and further development of in vitro plantlets, whereas no effect of CM& PGR on initial differential stages of ZEs whereas on further development of in vitro plantlets there was a significant effect of CM. The best culture medium for the growth and development of plantlets was N6 medium supplemented with PGR and AC which shows vigorous growth (18.58 cm) than the remaining media formulations.

Keywords: *Elaeis guineensis*; oil palm; zygotic embryo; germination; activated charcoal.

INTRODUCTION

Oil palm is a perennial crop species with highest (4-6 tons of oil ha^{-1}) edible oil yield, it has a vast usage in the pharmaceutical, cosmetic and biofuel industries [1,2]. Thus it's commercial importance accelerating the breeding of high yielding elite palms. Thus increasing demand must be accompanied by availability of plantlets, but as it is a monocotyledonous species with a single

growing apex, the plant cannot be multiplied vegetatively. And conventional seed propagation requires a long duration (105-120 days) for the asynchronous germination by the dry heat scarification method, still with low germination rates (30%) [3,4], whereas in vitro propagation of oil palm has been shown to overcome these limitations by increasing the number of plants produced in a short time [5], though there are many reports of oil palm tissue culture using various explants [6,7], zygotic embryos are the most desirable source of explants, because of their abundant availability, convenience of transportation, and more responsive to *in vitro* culture than any other explants [8]. Although several reports available, still there is a lack of an efficient, reliable and rapid regeneration system for this crop, as stated by the Corley and Tinker [9] "the oil palm tissue culture protocols have been continuously refined, resulting in various success rates". Standardization of the efficient in vitro regeneration protocol necessitates the suitable basal culture medium which will provide all the required nutrients.

In general culture media (CM) plays a vital role in the germination of embryo, and on the basis of culture, Murashige Skoog Medium (MS) is widely used as a basal medium for proliferation in several crops. Several researchers have reported the use of basal MS for culturing of mature zygotic embryo(MZEs) [10,11] and Chu et al.'s [12] N6 medium which is now widely using in several crops including oil palm [13] where greater plantlet regeneration on modified N6 was observed. In addition Eeuwans's [14] Y3 medium was the first to work in palm tree tissue culture, using zygotic embryos as explants and has been reported to germinate oil palm MZEs [15,16,17].

Plant growth regulators (PGR) are also may influence the germination and morphogenesis [11] and one more vital factor which seems to influence the growth and development of explants is activated charcoal (AC). Where AC has been proven in several crops to enhances the germination percentage and morphogenesis [18,19,20,21,22,23]. Hence the objective of the present study is to examine the effects of activated charcoal (AC), comparative suitability of the three different basal culture media and the role of plant growth regulators on the different morpho developmental stages of oil palm zygotic embryos.

MATERIALS AND METHODS

Plant Materials

The dura seeds used in this study were obtained from the elite palms seed garden mother block at ICAR-Indian Institute of Oil Palm Research, which have been evaluated and selected for the advanced breeding material and hybrid seed production.

Preparation of Explants

Open pollinated fresh fruit bunch of mature elite oil palm was harvested and fruits were depericarped using a depericarper in the seed production lab of ICAR- IIOPR, shell was removed by cracking with hammer to release the kernel. Obtained kernels were surface sterilized by adding few drops of Tween 20 and then immersing the kernels in fungicide solution of (1% Carbendazim & 1% Mancozeb). And soaked in distilled water for 5 days, for attaining the required moisture content of the zygotic embryo.

Then the kernels were washed repeatedly with Tween 20 solution (10drops/100ml v/v) for 15 minutes and washed with running tap water. They were then washed by fungicide sol (1% Carbendazim & 1% Mancozeb).They were then soaked in ethanol for 1 min and then washed with 20% NaOCl sol for 20 minutes. Then kernels were halved and embryos were surface disinfected with 20% (v/v) NaOCl for 20 minutes and then washed with the sterile distilled water for three times.

Culture Medium

Three types of Basal Culture medium MS [24], N6 [12] & Y3 [14] (Table 1) supplemented with or without PGR - GA_3, BAP and NAA at 0.2 mg L^{-1} [11,25] and with or without Activated Charcoal(2.0g/L) were used. The pH of the medium was adjusted to 5.8 followed by an addition of 8.0g L^{-1} agar prior to sterilization at 121°C for 20 min.

Culture and Growth Conditions

Excised embryos were cultured on 10 ml medium in test tubes. All culture tubes with explants were incubated in the dark growth room at a temperature of 25±2°C for 20 days and different developmental stages of embryos were recorded on 10 days intervals for twice. Later they were shifted to 16h photoperiod light chamber at a temperature of 25±2°C for up to 90 days and shifted to green house for the acclimatization.

Statistical Analysis

The experimental design was three factorial treatment combinations of culture media with & without plant growth regulators (PGR) and with & without Activated charcoal (AC) arranged in a randomized complete block design. Statistical analysis was done using WASP2, a web based software developed by ICAR-Central Coastal Agricultural Research Institute, Goa, India.

RESULTS AND DISCUSSION

Based on the ontogeny of oil palm zygotic embryos during the initial days of culture, 5 different developmental stages (Fig. 1) were classified [26,27] where stage0-no response, stage1-turgescence, stage2-haustorium initiation, stage3-geotropic curvature, stage4-shooting and stage5-rooting. After first 10 days of culture, meristematic pole of the embryo had started growing, reaching a sub conical shape which corresponds to stage3-geotropic curvature this may be the first sign of viability & germination but no significant effect (Table 3) of CM and PGR was observed on different morpho developmental stages of oil palm ZEs even after 20 days of culture (Table 3), similarly no significant differences for culture response were found between MS &Y3 in Jucara cultures [28], in oil palm similar result was reported by Padua et al. [29 & 30] where both MS&Y3 has shown statistically insignificant effect. Whereas activated charcoal also has shown similar trend up to stage2 (Table 3) during the initial 10 days of culture but on stage3 it has shown significant effect (Table 3) with AC treatments (27.005) when compared to without AC treatments (9.579). Same trend was observed in the stage4 (Table 3) after 20 days of culture on with AC treatments (61.654) when compared to without AC treatments (16.406). Kingsley et al. [31] and Alves et al. [32] also reported the similar results where all the CM in which AC was supplemented showed a better germination & development whereas embryos cultured on the medium without AC showed minimal development.

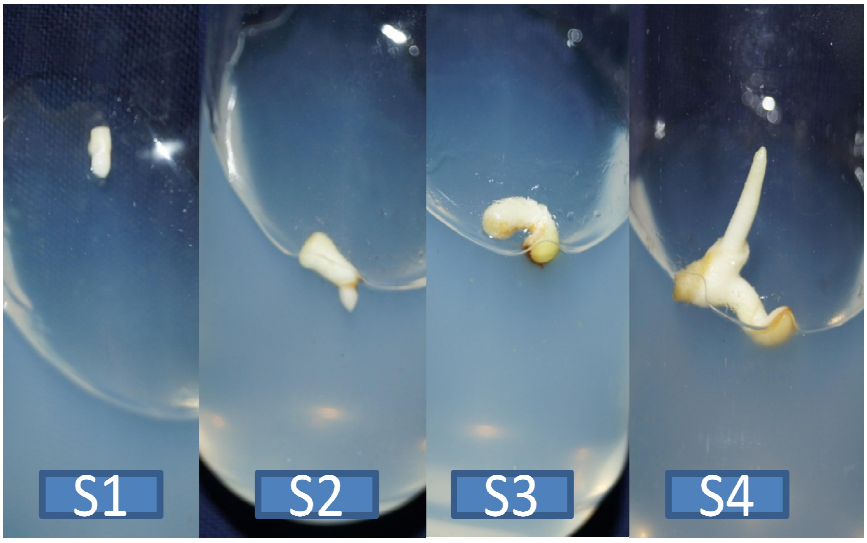

Fig. 1. Different developmental stages of oil palm zygotic embryos within 20 days of culture

Table 1. Composition of culture media used in the present study

Nutrients	Composition (mg L^{-1})		
	MS	N6	Y3
MACRO			
$(NH_4)_2 NO_3$	1650	-	-
$MgSO_4.7H_2O$	370	3.74	-
KNO_3	1900	56.6	5050
KH_2PO_4	170	8	617.5
NH_4Cl	-	-	1337.5
$NaH_2 PO_4.H_2O$	-	9.26	780
KCl	-	-	3730
$(NH_4)_2 SO_4$	-	9260	-
CALCIUM			
Cacl2	440	8.3	735
MICRO –I			
H_3BO_3	6.2	-	78
$MnSO_4 4H_2O$	22.3	4.4	28
$ZnSO_4 7H_2O$	8.6	1.5	18
KI	0.83	0.083	20.8
$Na_2MoO_4 2H_2O$	0.25	-	25 mg
MICRO – II			
$FeSo_4 7H_20$	27.8	1.39	-
Na_2 EDTA $2H_2O$	37.2	1.86	-
MICRO – III			
$CuSO_4 7H_2O$	0.025	-	-
$CoCl_2 6 H_2O$	0.025	-	-
ORGANICS			
Nicotinic acid	0.5	0.5	1
Pyridoxine-HCl	0.5	0.5	1
Thymine-HCl	0.1	1.0	2
m-Inositol	100	-	100
Glycine	200	2.0	200
Sucrose	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}
Agar	8 g L^{-1}	8 g L^{-1}	8 g L^{-1}

Table 2. Treatments used in this study which consists of MS, N6&Y3 basal medium supplemented with the required amount of PGR (NAA, BA and GA_3) and with or without Activated Charcoal (AC)

Treatment	Culture medium composition(mg L^{-1})
T1	MS+2000AC+0.2 NAA+0.2 BAP+0.2 GA_3
T2	MS+2000AC+No PGR
T3	MS+ No AC+0.2 NAA+0.2 NAA+0.2BAP+0.2G
T4	MS+No AC+No PGR
T5	N6+2000AC+0.2 NAA+0.2 BAP+0.2 GA_3
T6	N6+2000AC+No PGR
T7	N6+ No AC+0.2 NAA+0.2 NAA+0.2BAP+0.2G
T8	N6+No AC+No PGR
T9	Y3+2000AC+0.2 NAA+0.2 BAP+0.2 GA_3
T10	Y3+2000AC+No PGR
T11	Y3+ No AC+0.2 NAA+0.2 NAA+0.2BAP+0.2G
T12	Y3+No AC+No PGR

As an embryos further developed, percentage survival (number of embryos that developed into plantlets) was recorded after 30 Days of culture. The percentage survival of the embryos were lower than the viability in all the treatments (Table 2). There were significant differences between viability and survival in the treatments of media containing AC and without AC. (Fig. 2).

The culture media with AC and supplemented with & without PGR treatments (T1, T2, T5, T6, T9&T10) has shown highest range of percentage (77.21- 64.42) of survival (Fig. 2), while in the remaining culture media, survival percentage was ranging (0-38.16).

Irrespective of PGR's presence in the media, without AC has shown browning of the embryo

and media which may be due to the presence of polyphenolic compounds, causes tissues to suffer oxidation, due to polyphenolic oxidase enzyme [20]. The oxidation of the exuded phenolics often results to browning which blocks uptake of nutrients with consequences ranging from retarded development to death of explants [33,34]. Hence the ZEs did not show browning of explants in all three culture media with activated charcoal (AC) when compared to all the media without charcoal. Generally during the initial stages of tissue culture, excessive polyphenols are produced, activated charcoal reduces the browning of palm explants and culture media and increased explants survival and organogenesis. This might be due to the activated charcoal which may be able to absorb the toxic brown/black pigments (phenol-like compounds and melanin) as well as other unknown clourless toxic compounds [35], so that it provides suitable environment for the growth of seedlings through a very fine network of pores with large inner surface area on which many substances can be adsorbed [18,36,37,38,39].

Inpeuy et al. [5] also reported the same, where he observed intense colour change in the culture media void of AC during the somatic embryogenesis of *in vitro* oil palm shoot generated from MZEs.

The morphology of the developing plantlets varied greatly according to the medium used after 30 days of culture (Figs. 3& 4), plantlets which developed from embryos cultured on N6 (T5&

Table 3. Effect of Culture media, activated charcoal and plant growth regulators on different stages of oil palm zygotic embryos

Treatments			10 days after inoculation				20 days after inoculation				
			Stage0	Stage1	Stage2	Stage3	Stage0	Stage1	Stage2	Stage3	Stage4
			Mean of main effects								
MS		M1	36.717	18.216	25.888	19.179	34.967	11.052	3.000	19.084	31.895
N6		M2	37.379	14.795	29.469	18.357	29.964	12.694	8.684	3.023	45.634
Y3		M3	43.158	22.505	16.997	17.340	45.435	11.574	0.208	3.222	39.560
AC		A1	32.325	18.905	21.765	27.005	26.866	3.912	1.980	5.586	61.654
NAC		A2	45.845	18.105	26.470	9.579	46.712	19.635	5.948	11.300	16.406
	PGR	P1	41.295	21.187	20.011	17.507	36.595	13.615	3.166	8.434	38.190
	NPGR	P2	36.875	15.824	28.224	19.076	36.983	9.932	4.762	8.452	39.871
		CV(%)	46.009	76.615	74.423	74.311	34.889	96.745	184.150	138.361	32.897
		CD at 5%(M)	19.790	15.603	19.753	14.959	14.125	12.535	8.034	12.856	14.130
		CD at 5%(A)	16.158	12.740	16.128	12.214	11.533	10.235	6.560	10.497	11.537
		CD at 5%(P)	16.158	12.740	16.128	12.214	11.533	10.235	6.560	10.497	11.537

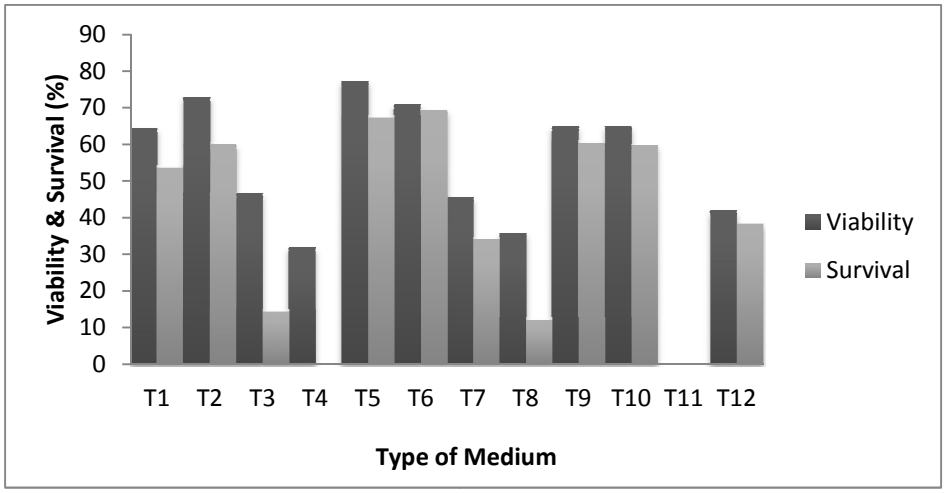

Fig. 2. Viability and survival of embryos cultured on different media 10 and 30 days after culture

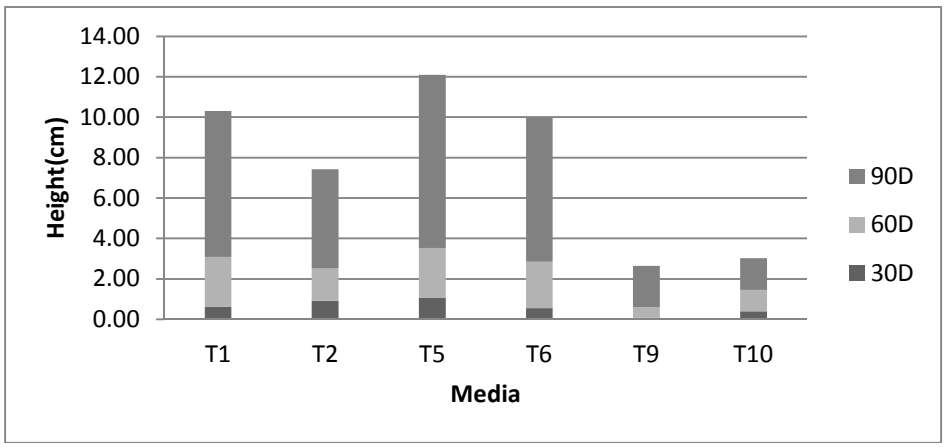

Fig. 3. Effect of media formulations on plant height in oil palm zygotic embryos after 30, 60 and 90 days of culture

T6) grew vigorously (18.58 & 7.21) followed by the embryos cultured on MS (T1& T2) and the embryos cultured on Y3 media (T9& T10) were having abnormal growth of 2.05 and 1.58 cm respectively (Figs. 3 & 4), a similar effect was observed by Pádua et al. [27]. Whereas the embryos cultured on without AC treatments (T3,T4,T7,T8,T11 and T12) regardless of CM and PGR showed minimal development and eventually died after 20 days of culture, this is an indication that AC is vital not only for germination but also for promoting the effect of organogenesis [18] this result also affirms the report of Van-Winkle and Pullman [39] who pointed out that apart from mere adsorption, AC demonstrates a greater plant survival, greater plant growth and improved plant quality and vigor (Fig. 2). The inclusion of PGR appeared to play a decisive role (T5&T1) offering an added advantage, similar to Suranthran *et al* [11], Samarina et al. [41], and Alves et al. [32] where low concentrations of auxin and cytokinin combinations are established to be effectual for the establishment of oil palm ZEs.

CONCLUSION

Based on the results obtained in this study on the growth and development of zygotic embryos of elite dura, it can be mentioned that culture media N6 & MS with AC is necessary to ensure the ability to early morpho differentiation and successful regeneration of the plantlets (Fig. 5). And the presence of PGR in the medium witnessed the further development of the plantlet.

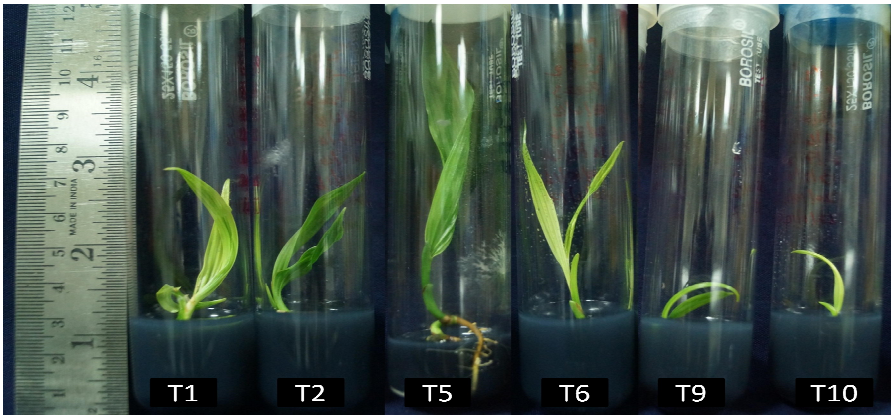

Fig. 4. Effect of culture media on plantlet development of oil palm 60 days culture

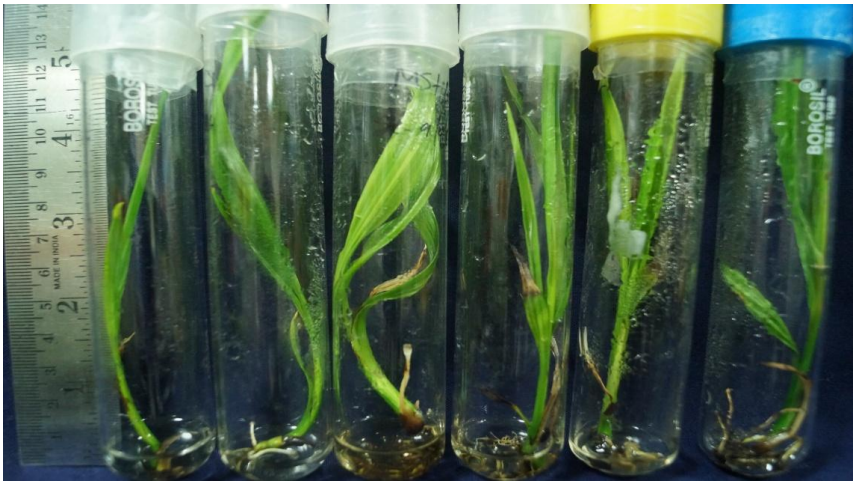

Fig. 5. Well developed plantlets obtained from the ZEs cultured on T5 medium after 90 days of culture

ACKNOWLEDGEMENTS

The authors thank the NMOOP, DAC, Ministry of Agriculture and Farmer Welfare, Govt. of. India for the financial support and fellowship.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Authors DSS, PNK and DR conceptualized the work. Authors DSS and BS investigated the study, author DSS wrote the original draft and authors PNK, DR and MSRK reviewed the draft. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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