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Effect of culture media, plant growth regulators and genotypes on growth and developmental stages of oil palm (*Elaeis guineensis* Jacq.) zygotic embryos

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ABSTRACT

Oil palm (*Elaeis guineensis* Jacq.) has become the largest traded vegetable oil in the world due to its innumerable uses and its huge economic importance. Which increases the requirement of quality seedling but the conventional seed production limits the commercial seed production with its long germination period and low germination rate. In this context *in vitro* germination of zygotic embryo could be a promising alternative. Hence in this study the effect of culturing media, plant growth regulators and genotypes were assessed on *in vitro* regeneration and plantlet development of oil palm zygotic embryos. Zygotic embryos of four elite genotypes were cultured on MS, N6 and Y3 media, with or without plant growth regulators (NAA, BA& GA_3-0.2mg/lt). There was no effect of culture media, plant growth regulators and genotypes on germination of zygotic embryos. Whereas on the growth and development of in vitro plantlets, there was a significant effect of culture media and genotypes. Where culture media N6 performs well and Y3 has shown more abnormality. In case of genotypes, genotypes G1 and G2 shown better performance when compared to genotypes G3 and G4. The effect of plant growth regulators on germination and plant growth was non significant.

Key words: Genotype, Germination, *In vitro*, Oil palm, Zygotic embryo.

Abbreviations: NAA: 1-Naphthaleneacetic acid, BA : 6-Benzylaminopurine, GA_3 : Gibberellic acid, PGR: Plant growth regulators, ZE : Zygotic Embryo.

INTRODUCTION

Oil palm is the highest oil yielding monocot perennial and monoecious crop. The crop contributes significantly to the world's growing edible oil requirement and the ever-increasing demand must be accompanied by availability of commercial scale plantlets. In oil palm, conventional propagation is only possible by seeds which has long germination period (1-3 years) and low germination rate (30%) (Martine *et al*.2009; Luis *et al*., 2010). Success of any crop improvement program necessitates the sound presence of wider spectrum of genetic variability (germplasm) especially in respect of crops like oil palm where narrow genetic base is the major constraint in achieving genetic variance through breeding. Hence attempts to conserve the selected mother palms as well as the variability for further use in breeding program are essential. But the conservation of promising material in field gene bank is resources demanding. As it is a monocot plant, this species cannot be propagated by micro cuttings. Thus, to improve germination and reduce time for establishing new plantings there is a need for developing alternative techniques. Hence only tissue culture can be used as a contrivance for optimizing embryo germination, though there are several reports on oil palm tissue culture using various explants sources (Teixeira *et al*.1993, 1994); zygotic embryos are the most enviable source of explants, because of their abundant availability and convenience of transportation and they are notably faster and more responsive to in vitro culture than other explants sources (Abdullah *et al*.2005). Successful culture of any explant requires a growth medium that can supply all the essential supplements, where Murashige and Skoog (MS) basal medium (1962) has been widely used as a culture medium for growth and development of oil palm zygotic embryos (Thawaro and Te-chato, 2010& Suranthran *et al*, 2011), Eeuwans's (1976) Y3 medium was the first to work in palm tree tissue culture, using zygotic embryos as explants (Silva,2002 and Steinmacher, 2005) and Chu *et al*. (1975) N6 medium which is now widely using in several crops including oil palm (Thuzar *et al*.2011). Engelmann *et al*. (1995) studied the cryopreservation of zygotic embryos and

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kernels of oil palm and reported the various stages of germination of oil palm zygotic embryos as per the classification made by Rabechault (1968). According to him "the different stages *viz* stage1-turgescence was corresponded to embryos which had rehydrated, increased in volume after transfer onto culture medium, thus becoming turgescent, stage II-geotropic curvature was corresponded to embryos whose haustorium had started growing ,stage III-nail shape, the meristematic pole of embryos had started growing, reaching a sub-conical shape".

Plant growth regulators (PGR) play a vital role in several crops to influence growth and morphogenesis as well as on oil palm (Suranthran *et al*.2011). Another factor which seems to influence growth and development of explants in culture is the genotypic variation (Kingsley *et al*, 2016, Alves *et al*, 2011& Martine *et al*, 2009). Hence the present study was therefore to evaluate the effect of culture medium, presence or absence of PGR and genotypes, envisaged to conserve the diversity of the oil palm and utilization of the conserved embryos necessitates the *in vitro* protocol wherever required.

MATERIALS AND METHODS

Plant materials: The four genotypes were from Dura mother palm block in ICAR-IIOPR seed garden where the material belongs to four D X D(Dura X Dura) crosses, 240D X 281D (G1) and 80D X 281D (G2),98C X 254D (G3) and 98C X 208 D(G4), which have been evaluated and selected for the advanced breeding material and hybrid seed production.

Preparation of explant: Mature oil palm open pollinated fresh fruit bunches of four genotypes were 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. Kernels obtained were surface sterilized by adding few drops of Tween-20 and then immersing the kernels in fungicide solution of (1% Carbendazim and 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 solution(1% carbendazim and 1% Mancozeb). They were then soaked in ethanol for 1 minute and then washed with 20% NaOCl sol for 20 minutes. Then kernels were halved and embryos were sterilized with 20% (v/v) NaOCl for 20 minutes and then washed with sterile double distilled water for three times.

Culture medium: Three types of Basal Culture medium *viz*., MS (Murashige and Skoog,1962), N6 (Chu *et al*.1 975) & Y3 (Eeuwens,1976) were supplemented with 30g/L (W/V) Sucrose, and with PGR and without PGR - GA_3, BA and NAA at 0.2 mg/L (Suranthran *et al*.,2011 and Chen *et al*, 2008) and Activated Charcoal(2.0g/L)were used. The pH of the medium was adjusted to 5.8 and added 8.0g/L of Agar prior to sterilization at 121 ^{o}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 ^{o}C for 30 days .Later they were shifted to 16 h photoperiod light chamber at a temperature of 25±2 ^{o}C for up to 90 days.

The number of germinated embryos was recorded. Embryos were considered viable when they had expanded and showed signs of Haustorium formation 10 days after culture. Further observations on embryo development were taken 10 days intervals. After 25 days of culture observations like germination, abnormality, shoot length and root induction were recorded and furthermore morphological characters including plant height, number of leaves were recorded after 30,60 and 90 days of culturing.

Statistical analysis: The experimental design was three factorial treatment combinations of culture media with or without plant growth regulators and genotypes arranged in a randomized complete block design and repeated twice.

RESULTS AND DISCUSSION

Effect of culture media, PGR and genotype on developmental stages of oil palm zygotic embryo: Stages of the embryos were defined according to their anatomical conditions, expansion of cotyledon, elongation or bending and the development of shoot and root. Based on Engelmann *et al*. (1995) we further classified the different developmental stages (Fig 1) of zygotic embryos where stage0-no response (Fig 1A), stage1-turgescence (Fig 1B), stage2-haustorium (Fig 1C) had started growing, stage3-geotropic curvature (Fig 1D), stage4-shooting (Fig 1E) and stage5-rooting (Fig 1F).

In first 10 days of culturing the zygotic embryos development were recorded up to Stage 3(Fig 1D) (no response to geotropic curvature/nail shape, which was corresponded to the meristematic pole of embryos had started growing, reaching a sub conical shape). And after 20 days of culturing ZEs were recorded up to Stage 4- shooting (Fig 1E). The pattern of oil palm zygotic embryo development and morphogenesis was confined up to expansion stage only without extreme shoot or root development during the first twenty days of culture. After inoculating the embryos in the culture media first 1 to 5 days there was no further development, hence the stage 0 was defined as no responsive, after 10 days and 20 days of culture there was no significant difference noticed among three culture media tested but there was a significant difference observed in with and without Plant growth regulators after 10days of culturing where with PGR shown higher significance than without PGR but after 20 days of culture there was no significant difference between with and without PGR

(Table 1).Which means only during first 10 days of culture without PGR cultured embryos moved to further stages. And where as in genotype there was a significant difference noticed on Stage0 where G4 was more significant in both after 10 days and 20 days of culturing and G3 was least significant in 10 days after culture but after 20days of culture, G1 was least significant which was on par with G2 and G3.which means except G4 all three genotypes were shown more speed of germination thus they have moved to further stages within 1 to 5 days of culture (Table 1).

When the embryo was becoming turgescent by absorbing the nutrients available in the culture media which was the basic sign of response was defined as "turgescence" corresponded to embryos which had rehydrated, increased in volume after transfer onto culture medium this might be due to early cell division which occurs in the procambium and in the parenchyma cells adjacent to vascular tissue, as a result these were newly added cell layers in the explants causing embryos to expand and this was categorized as a Stage1 where there was a significant effect noticed in different culture media after 10 days of culturing and Y3 media was found to be more significant where similar result was found by Muniran *et al*. (2008). And followed by least significant N6 media but after 20 days of culturing the effect of culture media were found to be non-significant (Table 1). In case of with and without growth regulators after both 10 and 20 days of culturing with PGR was more significant than without PGR (Table 1). And with respect to genotype only after 10 days of culturing has significant difference where G1 was more significant and G4 was lease significant (Table 1).

Stage 2 was defined as starting and extension of Haustorium which plays an active role in the hydrolysis of lipids and mobilization of reserves (Verdeil and Hocher, 2002). After 10 days of culturing; only culture media PGR and genotype has shown significant difference and 20 days after culturing no significant difference was noticed in stage2. After 10 days of culturing culture media N6 was more significant this was on par with MS and least significant in Y3, similar results were observed in Supawadee Thawaro and Sompong Te-chato (2010). Along with or without plant growth regulators, without plant growth regulators was more significant. Whereas among genotypes G3 (39.30%) was more significant and G4 (3.96%) was least significant in Stage 2 (Table 1).

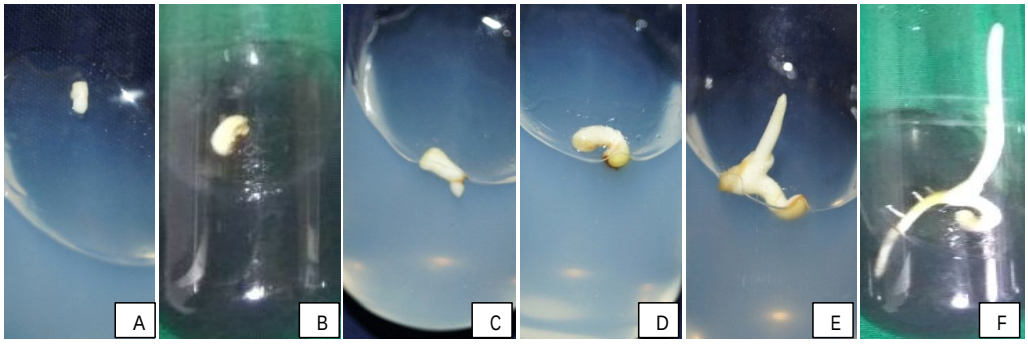

Fig 1: Different Developmental Stages of Oil Palm Zygotic Embryos, A.Stage0-No Response, B.Stage1-Turgescence,C.Stage2-Haustorium Initiation, D.Stage3-Geotropic Curvature, E.Stage4-Shooting, F.Stage5-Rooting.

Table 1: Effect of media, growth regulators and genotype on the relative proportion (percentage) of developmental stages of *in vitro* germination of oil palm zygotic embryos on 10th and 20th days of culturing.

		10 days after culturing				20 days after culturing				
		Stage0	Stage1	Stage2	Stage3	Stage0	Stage1	Stage2	Stage3	Stage4
	Treatments	Mean of main effects								
MS	M1	31.93	18.812	22.821	26.437	27.846	3.526	2.867	9.111	56.649
N6	M2	34.454	13.173	22.943	29.43	23.736	2.184	2.657	3.148	68.274
Y3	M3	30.59	24.73	19.532	25.148	29.016	6.027	0.417	4.5	60.041
PGR	P1	34.694	21.88	13.173	30.253	26.521	4.621	2.761	5.797	60.3
NPGR	P2	29.955	15.93	30.358	23.757	27.211	3.204	1.2	5.376	63.009
240D X 281D	G1	28.444	57.658	10.668	3.23	17.376	2.024	1.792	2.589	76.219
80D X 281D	G2	25.987	1.861	33.176	38.976	20.946	3.542	0.918	5.791	68.804
98C X 254D	G3	19.661	14.586	39.301	26.451	19.552	5.834	3.505	7.578	63.531
98C X 208D	G4	55.206	1.515	3.916	39.363	49.591	4.249	1.706	6.388	38.065
	CV(%)	20.023	29.914	17.508	13.382	36.868	148.089	225. 525	79.306	19.674
	CD at 5%(M)	4.735	4.137	2.788	2.643	7.245	4.238	3.267	3.241	8.873
	CD at 5%(P)	3.866	3.378	2.276	2.158	5.916	3.46	2.667	2.646	7.245
	CD at 5%(G)	5.467	4.777	3.219	3.052	8.366	4.894	3.772	3.742	10.246

The meristematic pole of the zygotic embryo when started growing and reached a sub conical shape which corresponded to geotropic curvature/nail shaped was categorized as stage3 while the cotyledon increases markedly in diameter and becomes green. The apex emerges from the side near the distal end and the root tip later emerges from the radical end. Culture media N6 (29.43%) was more significant after 10 days culture and Y3 (5.148%) was least significant but after 20 days of culture contrary to previous results MS culture media was more significant and N6 (3.148%)was least significant which was on par with Y3 (4.5%) (Table 1).After 10 days of culture with PGR (30.253%) was more significant than with PGR but after 20 days of culture without PGR (5.79%) was more significant (Table.1). Whereas among genotypes after 10 days of culture G4 (39.363%) was more significant which was on par with G2 (38.976%), but after 20 days of culturing there was no significant effect on stage3 (Table 1).

After 15 to 20 days of culturing the shooting occurs in the proximal region which represents the embryonic axis, containing a plumule with primordial leaves that surround the apical meristem (Silva *et al*., 2014). The development of zygotic embryo leaf primordia was observed in the proximal region, which was categorized as stage4 and no shooting was observed within 10 days of culture thus there was no data in this duration. Whereas after 20 days culturing, culture media N6 has more significance (68.274%) which was on par with the Y3(60.041%) (Table 1).With and Without plant growth regulators has no significant effect on shooting as stated by Termizi *et.al*, (2014). But whereas genotype has significant effect on shooting, where G1 was more significant (76.219%) which was on par with G2 (68.804%) and G4 was least significant on stage4 (Table 1) a similar trend was obtained by Thawaro and Te-chato (2010).

In over all stages Culture media has significant effect in both 10 &20 days after culturing where culture media N6 performed well this result suggested that the nutrient composition of N6 may more suitable for cell growth and cell differentiation of callus than the nutrient composition of MS and Y3 similar work was reported by Thuzar *et al*. (2011) and Abdullah *et al*. (2005), but whereas with and without PGR and genotype has shown significant effect only initial 10 days of culturing where varying results were observed in case of PGR but in case of genotype, G3& G4 were least performed and G1& G2 were performed well, but after 10 days of culturing there was no significant effect of PGR and genotype on different developmental stages of oil palm zygotic embryos.

Effect of culture media, PGR and genotype on germination and abnormality of oil palm zygotic embryos: Zygotic embryos were considered surviving only after 20 days when any sign of regrowth was observed *viz*., normal development, root pole& shoot or cotyledon development, or else any volume expansion (Camillo *et al*., 2009). Hence the germination percentage was estimated after 25 days of culturing by counting the number of embryos which were on or above stage 2. There was no significant effect of culture media (Table 2), a similar result was observed by Pádua *et al*. (2014a) where the embryos of 100 days post-anthesis in MS medium showed the best result for germination rate which did not differ statistically from Y3 medium (Table 2). And even no significant effect of PGR and genotype on germination where more than 90% germination was achieved in all the treatments (Table 2) similar to results obtained by Daniel *et al*. (2003). Zygotic embryos with abnormal behavior like germinated without plumule and/or shoot and/or root pole and/or callusing were treated as abnormal seedlings after 25 days of culturing. Culture media Y3 was more significant with highest per cent abnormality (71.170%) and least abnormality was recorded in N6 (41.778%) which was on par with MS (44.019%) (Fig 2 & Table 2). In case of with & without PGR, with PGR shows more abnormality (59.992%) than without PGR (44.653%) which didn't differ statistically (Fig 2 & Table 2). Whereas among genotypes G4 was produced more abnormal seedlings (71.012%) which was on par with the G3 (51.975%) and G1 (50.147%), and least abnormality was recorded in G2 (36.157%) (Fig 2 and Table 2).

Effect of culture media, PGR and genotype on shoot length and root induction after 25 days of culturing oil palm zygotic embryos: Shoot length of *in vitro* seedlings were recorded after 25 days of culture and differentiated in to three categories *viz*., ≤1cm,1 to 3cm and ≥3cm. Culture media MS (69.545%) was more significant and N6 (60.755%) was least significant but there was no statistical significance. Hence there was no effect of culture media on ≤ 1cm shoot length seedlings. Culture media Y3 (39.105%) was produced more 1 to 3 cm shoot length seedlings which was on par with N6 (38.284%) and MS (22.492%) was produced least number of seedlings. And culture media MS was produced more (7.963%) ≥3cm shoot length seedlings, and no seedlings were produced in Y3 culture media ≥3cm shoot length which was on par with N6 (0.962%) (Table 3).

In case of with and without PGR shown similar effect on ≤ 1cm, ≥ 3cm shoots length seedlings where with PGR produced more number of seedlings than without PGR. But in contrary to these results without PGR produced more number of 1 to 3 cm shoot length seedlings. Whereas among genotypes G4 was produced more (76.369%) ≤ 1cm shoot length seedlings which was on par with G1 (68.907%) and G3 (66.636%) and least number of seedlings were produced in G2 (43.015%), More 1 to 3 cm shoot length seedlings were produced by G2 (48.908%) which was on par with G3 (32.723%) and least number of seedlings were produced by G4 (22.798%) which was on par with the G2 (28.745%). And in case of ≥ 3cm shoot length seedlings G2 was

produced more number of seedlings (8.077%) and least number of seedlings were produced by G3 (0.641%) which was on par with G4 (0.641%) and G1 (2.348%). By these results we can say that 43 to 76% seedlings were on ≤ 1cm shoot length range and G4 genotype was mostly retained in ≤ 1cm range only and genotype G2 Produced more ≥ 3cm shoot length seedlings (Table 3).

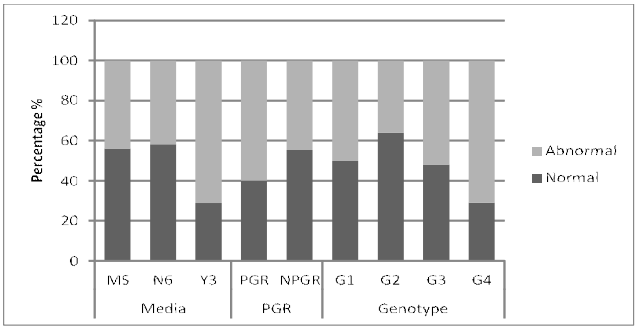

Fig 2: Effect of media,growth regulators and genotypes on abnormality of *in vitro* germinated zygotic embryos.

After 25 days of culture 1 to 18% seedlings only produced rooting where culture media N6 produced more rooted seedlings (12.32%) and lowest rooted seedlings were produced in MS (5%) followed by Y3 (9.43%) but all were statistically on par (Table 3), which was a similar result obtained by Cleber and Maisa (2012), where there was no significant effect of culture media on root induction. But with & without PGR shows significant effect on rooting where without PGR produced more rooted seedlings (17.04%) (Table 3), whereas among genotypes G3 produced more rooted seedlings (18.45%) which was on par with G4 (9.52%) and G2 (7.3%) and least rooted seedlings were produced by G1 (1.76%) (Table 3).

Effect of culture media, PGR and genotype on height of the seedlings and number of leaves after 30, 60 and 90 days of culture: The growth parameters *viz.*, seedling height, number of leaves were recorded at 30,60 and 90 days after culture. Height of the seedlings after 30,60 and 90 days of

Table 2: Effect of media, growth regulators and genotype on the relative proportion (percentage) of germination, and abnormality of *in vitro* germinated oil palm zygotic embryos on 25th day of culturing.

	Treatments	Ger	Unger	Normal	Abnormal
			Mean of main effects		
MS	M1	90.846	9.15	55.981	44.019
N6	M2	93.180	6.82	58.222	41.778
Y3	M3	90.589	9.41	28.830	71.170
PGR	P1	91.952	8.05	40.008	59.992
NPGR	P2	91.125	8.88	55.347	44.653
240D X 281D	G1	91.913	8.09	49.853	50.147
80D X 281D	G2	93.263	6.74	63.843	36.157
98C X 254D	G3	92.963	7.04	48.025	51.975
98C X 208D	G4	88.014	11.99	28.988	71.012
	CV(%)	10.867	117.55	55.276	50.369
	CD at 5%(M)	7.276	7.28	19.278	19.278
	CD at 5%(P)	5.941	5.94	15.741	15.741
	CD at 5%(G)	8.402	8.40	22.261	22.261

Table 3: Effect of media, growth regulators and genotype on the shoot length and Root induction percentage of *in vitro* germinated oil palm zygotic embryos on 25th day of culturing.

	Treatments	1cm	1-3cm	3<cm	Rooted	Unrooted
				Mean of main effects		
MS	M1	69.545	22.492	7.963	5.00	95.00
N6	M2	60.755	38.284	0.962	12.32	87.68
Y3	M3	60.895	39.105	0.000	9.43	89.79
PGR	P1	70.769	25.909	3.322	1.48	98.53
NPGR	P2	56.694	40.678	2.628	17.04	83.27
240D X 281D	G1	68.907	28.745	2.348	1.76	98.24
80D X 281D	G2	43.015	48.908	8.077	7.3	94.37
98C X 254D	G3	66.636	32.723	0.641	18.45	81.55
98C X 208D	G4	76.369	22.798	0.833	9.52	89.44
	CV(%)	33.746	61.860	154.100	210.25	21.18
	CD at 5%(M)	15.732	15.066	3.353	14.24	14.08
	CD at 5%(P)	12.845	12.301	2.738	11.63	11.5
	CD at 5%(G)	18.166	17.396	3.872	16.44	16.26

culture recorded same significant effect among culture media, where culture media N6 has more significant growth followed by MS media and least growth was observed in Y3 culture media (Table 4). In contrary to Sumanthran *et al*. (2011) and Thawaro and Te-chato (2010) with &without PGR has no significant difference after 30 and 60 days of culture on height of the seedlings but whereas after 90 days of culture a significant difference was noticed in with PGR treatment (Table 4). And regarding genotype after 30, 60 and 90 days of culture, G2 was more significant which was followed by G1 and least significant in G4 which was on par with G3 (Table 4).

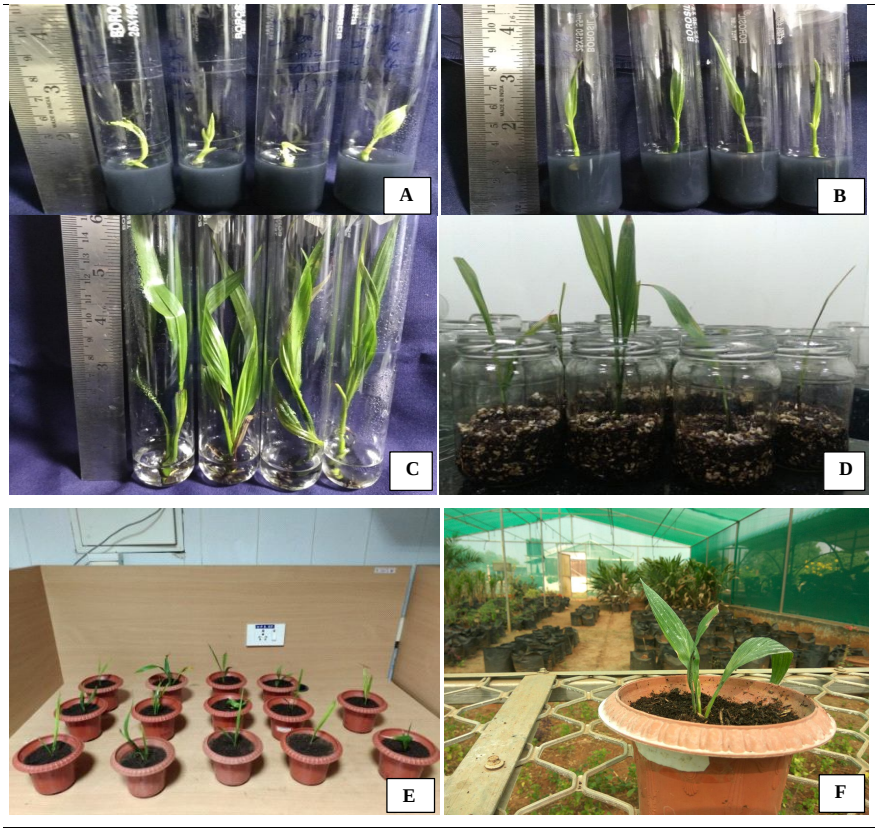

Fig 3: Oil palm zygotic embryo's *in vitro* germinated plantlets growth at different stages. A. *In vitro* generated plantlets from ZEs after 30 days of culture, B. *In vitro* generated plantlets from ZEs after 60 days of culture,C. *In vitro* generated plantlets from ZEs after 90 days of culture, D.Plantlets in Primary hardening media(Soilrite, Perlite, Cocopeat and Vermiculite-1:1:1:1),E. 90 days old plantlets transferred to earthen pots,F.150 days old *vitro* plantlet under acclimatization in green house.

Table 4: Effect of media, growth regulators and genotype on height and no of leafs of the seedlings on 30,60 and90 days of culture.

	Treatments	30Days		60Days		90Days	
		Ht	NoL	Ht	NoL	Ht	NoL
		Mean of main effects					
MS	M1	0.763	0.938	2.038	1.563	6.063	2.313
N6	M2	0.813	1.000	2.375	1.875	7.856	2.813
Y3	M3	0.238	0.625	0.788	1.063	1.813	1.875
PGR	P1	0.588	0.750	1.817	1.333	5.946	2.083
NPGR	P2	0.621	0.958	1.650	1.667	4.542	2.583
240D X 281D	G1	0.850	0.917	2.383	1.833	9.108	3.167
80D X 281D	G2	0.925	0.917	2.525	1.833	8.825	3.167
98C X 254D	G3	0.367	0.833	1.150	1.250	1.783	1.583
98C X 208D	G4	0.275	0.750	0.875	1.083	1.258	1.417
	CV(%)	25.827	29.689	30.072	36.778	34.134	35.652
	CD at 5%(M)	0.114	0.186	0.381	0.404	1.309	0.609
	CD at 5%(P)	0.093	0.151	0.311	0.329	1.069	0.497
	CD at 5%(G)	0.132	0.214	0.440	0.466	1.512	0.703

Number of leaves grown in seedlings after 30,60 and 90 days of culture recorded same significant difference among culture media, where culture media N6 was produced more significant number of leaves followed by MS media and least number of leaves were produced in Y3 culture media (Table 4), a similar effect was observed in the study conducted by Pádua *et al*. (2014b) in which, no increase in number of leaves in plants grown in Y3 and MS medium .With respect to with &without PGR, a significant difference recorded after 30,60 and 90 days of culture on leaf production where a significant difference was noticed in without PGR treatment (Table 4). And regarding genotype after 30, 60 and 90 days of culture, G1 and G2 were produced more number of leaves than G3 and G4 (Table 4).

After 90 days of culturing all the *in vitro* plantlets (Fig 3C) with well formed roots were transplanted to soil less hardening media consisting Soilrite, Perlite, Cocopeat and Vermiculite in the 1:1:1:1 ratio and grown in controlled environment (Fig 3D) for 60 days and after transplanted to potted soil (Fig 3E), plantlets (Fig 3F) were shifted to greenhouse for further acclimatization.

CONCLUSION

The results of the present study showed that the culture media and genotype played essential role for successful regeneration of plantlets from oil palm zygotic embryos. And no effect was observed in case of PGR, regardless of whether supplemented or not. Culture media N6 performed well when compared to MS &Y3.Whereas in case of genotypes both G1 &G2 have a high regeneration potential *in vitro* than G3&G4 genotypes .Studies are currently going on for a better acclimatization, seedling vigour and survival percentage in the primary nursery.

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