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Embryogenesis of 'Albescens'-type of Oil Palm (*Elaeis guineensis* Jacq.) in Eeuwens Medium

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Abstract

The growth response of zygotic embryos of 'Albescens'- type of 'tenera' oil palm (*Elaeis guineensis* Jacq.) using Eeuwens medium, supplemented with 1-Naphthalene Acetic Acid (NAA) and 6-Benzylaminopurine (BAP) was investigated. The respective concentrations (0.00 mg/L, 0.05 mg/L, 0.10 mg/L) of NAA and BAP were used singly and in combination. Zygotic embryos extracted from the 'Tenera-albescens' type of oil palm were sterilized and inoculated in Eeuwens media containing the various combinations of growth regulators. The cultures were incubated in the dark room at 26°C, 50-60% relative humidity and later transferred to the light room at 26°C, 50-60% relative humidity at 16 hr photoperiod. Subcultures were carried out at intervals of 4 weeks. Data were collected on days to shoot and root emergence, shoot length at 3 weeks after inoculation (WAI), root length and plant height at 4, 6, 8 and 10 WAI, number of leaves and roots at 10 WAI. The results showed that the concentrations of NAA and BAP singly and in combinations had significant ($p < 0.05$) effect on *in vitro* plant regeneration and plantlet development. However, synergistic effect of the two plant growth regulators as supplements were optimal for all growth parameters measured.

Key words: *Elaeis guineensis*, 'Albescens', dormancy, germination, Eeuwens, in vitro*

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Introduction

World demand for vegetable oil is much on the increase, from 100 million tonnes in 2005 to an estimated 150 million tonnes in 2020 (Ataga and Vossen, 2007). As the world population continues to grow and cost of living increases in many developing countries, the role of oil palm as a source of relatively inexpensive and versatile edible oil is therefore expected to become more prominent. The oil palm (*Elaeis guineensis* Jacq.) belongs to the Arecaceae family and originated from West Africa (Hartley, 1988). It is the major source of vegetable oil in the world and the first plant used in the production of vegetable oil commercially in the international market (Carvalho et al., 1997). The oil palm is a tropical species that grows mainly in hot, humid climate with abundance of rain. It is a perennial monocot with a long generation period of about 20 years. Mature palms are single-stemmed and grow to 20m tall (Carvalho et al., 1997).

There is a basic classification of *E. guineensis* varieties based on thickness of the shell as: 'Dura', 'Pisifera' and 'Tenera' (Opeke, 1992). Furthermore, oil palm is also classified based on fruit colour into: 'Albescens', 'Virescens', 'Nigrescens' and 'Idolatrica' (Seng et al., 2007). This classification is based on fruit

characteristics under monogenic control and includes presence or absence of anthocyanin in the upper fruit exocarp and carotene in the mesocarp. Extensive work has been done on 'Dura', 'Pisifera' and 'Tenera' fruit forms but on the basis of pigmentation, little research focus has been given to oil palm. Palm oil is used for a wide variety of industrial products. An essential component of these production processes is the bleaching of the oil to remove the carotene pigment. 'Albescens'-type however, is an extremely rare fruit-type, with low carotene in the mesocarp, thus, it could reduce the cost of bleaching particularly in the industrial use of palm oil.

Oil palm plant is normally propagated by seeds and seed germination is usually poor due to the long dormancy period. It requires a long time to germinate, 1-3 years and also shows low germination rate – approximately 30 % (Luis et al., 2010). Conventional vegetative propagation is usually not possible, because the oil palm has a single terminal meristematic point. Thus, there is a need to develop techniques that could improve propagation and reduce time for establishing seedlings. Tissue culture proffers a veritable tool for optimizing embryo germination and possible micropropagation of *E. guineensis* through tissue isolation and cultivation under aseptic condition. The potential of mass production of 'Albescens' seedlings through micro-propagation is very promising and would aid to conserve and retain its genetic integrity and purity. It is noteworthy that germination of zygotic embryo is greatly influenced by *in vitro* culture media composition. Many authors have reported the use of Murashige and Skoog (MS) basal medium as a standard culture medium for growth and development of plants in vitro (Chourykaew and Kanchanapoom, 1996; Komai et. al., 1996; Thawaro and Te-chato, 2010; Suranthran et. al., 2011); thereby giving less attention to other media such as Eeuwens' medium (Y3), Blayde's medium, White's medium, Blake's medium, Medium 73 and a whole lot more. Eeuwens medium has been used to germinate oil palm mature zygotic embryos (Chourykaew and Kanchanapoom, 1996). Normally, plants regulate their growth by synthesising phytohormones. However, under *in vitro* conditions, synthetic growth regulators are applied to ensure optimal growth of the plant cells and tissues. Plant growth regulators play an essential role in determining the developmental pathway of plant cells and tissues in culture medium (Hussian et al., 2012). The auxins, cytokinins and gibberellins are most commonly used plant growth regulators. The type and concentration used depend mainly on the species of the plant, the tissue or organ culture and the objective of the experiment. The objective of this study was therefore, to determine for the first time, the efficacy of Eeuwens medium supplemented with the plant growth regulators, 1-Naphthalene Acetic Acid (NAA) and 6-Benzylaminopurine (BAP) on growth response of zygotic embryos of 'Albescens'-type of 'Tenera' oil palm.

Materials and Methods

Plant materials

This study was carried out in the Plant Tissue Culture Laboratory of the Nigeria Institute for Oil Palm Research (NIFOR), Benin City, Edo State, Nigeria. Seeds from harvested bunches of 'Tenera-albescens'-type (Plate 1) were obtained from the Plant Breeding Division of the Institute. Zygotic embryos from these seeds were extracted and used for the study.

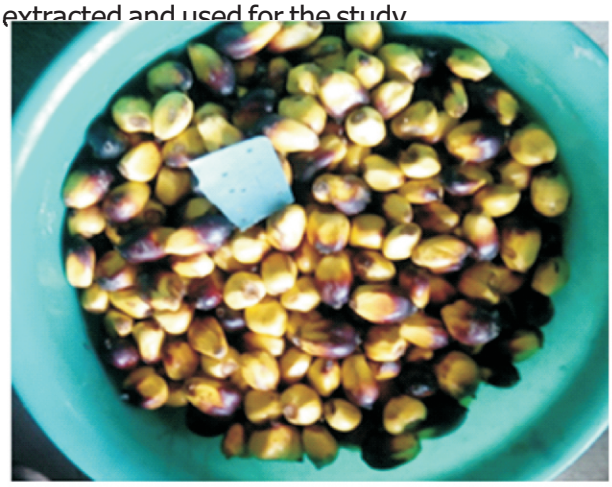

Plate 1: Ripe 'Albescens' fruits

Source: Nigeria Institute for Oil Palm Research (NIFOR)

Culture media preparation

study. The stock solutions for macro elements, micro elements A, micro elements B, iron source and organic nutrients of this medium were prepared by weighing out the salts and dissolving them in distilled water separately before mixing, in order to avoid the formation of complex insoluble salts. Following the preparation of the stock solutions, the preparation of the complete media was carried out using the media preparation sheet protocol as adapted from Dixon (1985). The medium was melted in a microwave for 20 minutes at 100°C. The melted medium containing the various concentrations and combinations of growth regulators were dispensed into duly labelled

Table 1: Treatments and treatment combinations

Treatment	Growth regulators		
	NAA mg/L	+	BAP mg/L
1	0.00		0.00
2	0.05		
3	0.10		
4	0.00		0.05
5	0.05		
6	0.10		
7	0.00		0.10
8	0.05		
9	0.10		

NAA - 1-Naphthalene Acetic Acid; BAP - 6-Benzylaminopurine

test tubes. These were autoclaved at 151 bs/m^2 pressure for 20 minutes at 121°C. After sterilization, the test tubes were shaken gently to uniformly disperse the activated charcoal in the medium bottles. They were allowed to cool and solidify at room temperature.

Extraction, surface sterilization and inoculation of embryo

The pericarp of the oil palm seeds was removed, followed by cracking with a hammer and removal of the endocarp. Seeds were sterilized by shaking in 70 % ethanol for 15 minutes, then in 3.5 % of sodium hypochlorite (NaOCl) for another 15 minutes. The embryos were extracted using a sterilized surgical blade. The extracted embryos were surface sterilized in 0.1 % sodium hypochlorite for 5 minutes. Traces of the sodium hypochlorite were removed by rinsing with distilled water thrice to get rid of the sterilant as this could become toxic at prolonged exposure. The embryos were later transferred into the test tubes containing the respective culture medium using sterilized forceps. The inoculated medium in the test tubes (cultures) were incubated in the dark room at 26°C, 50 to 60% relative humidity until emergence of shoot or root and transferred to the light room at 26°C, 50 to 60% relative humidity and 16 hours of photoperiod. Subcultures were carried out at the interval of 4 weeks.

Growth medium and the plant growth regulators treatments

The treatments consisted of Eeuwens medium supplemented with 0 mg/L, 0.05 mg/L, and 0.10 mg/L concentrations of NAA and BAP respectively. The treatments were administered alone and in all combinations as shown in Table 1.

Data collection

Data were collected on time of shoot and root emergence, shoot length at 3 weeks after inoculation (WAI), root length and plant height at 4, 6, 8 and 10 WAI, number of leaves and number of roots at 10 WAI.

Experimental design and statistical analysis of data

The experiment was carried out as a completely randomized design (CRD) with four replicates. The data were subjected to analysis of variance (ANOVA) using Genstat statistical software (12th Edition). Means were separated using Student Newman Keuls Test (SNKT) at 5 % probability level.

Results and Discussion

Swelling of the embryo was observed at

10 days after inoculation (DAI) (Plate 2b), followed by the formation of haustorium and the embryonic axis began to turn green (Plate 2c) leading to the emergence of plumule from the shoot apex within 14 days of culture and the shoot elongation at 3 WAI. Root emergence was observed after approximately 16 days of culture and the root was visible at 4 WAI; complete plantlet formation was observed one month after culture. Primary leaves and roots were observed at 10 WAI. This pattern of growth was also observed by Thawaro and Te-chato (2010) and Suranthran et al., (2011). Danson et al. (2009) also reported that embryos were considered germinated when the plumule sprouts and the radicle shows sign of emergence. Plates 2d-g showed that plant height and root length

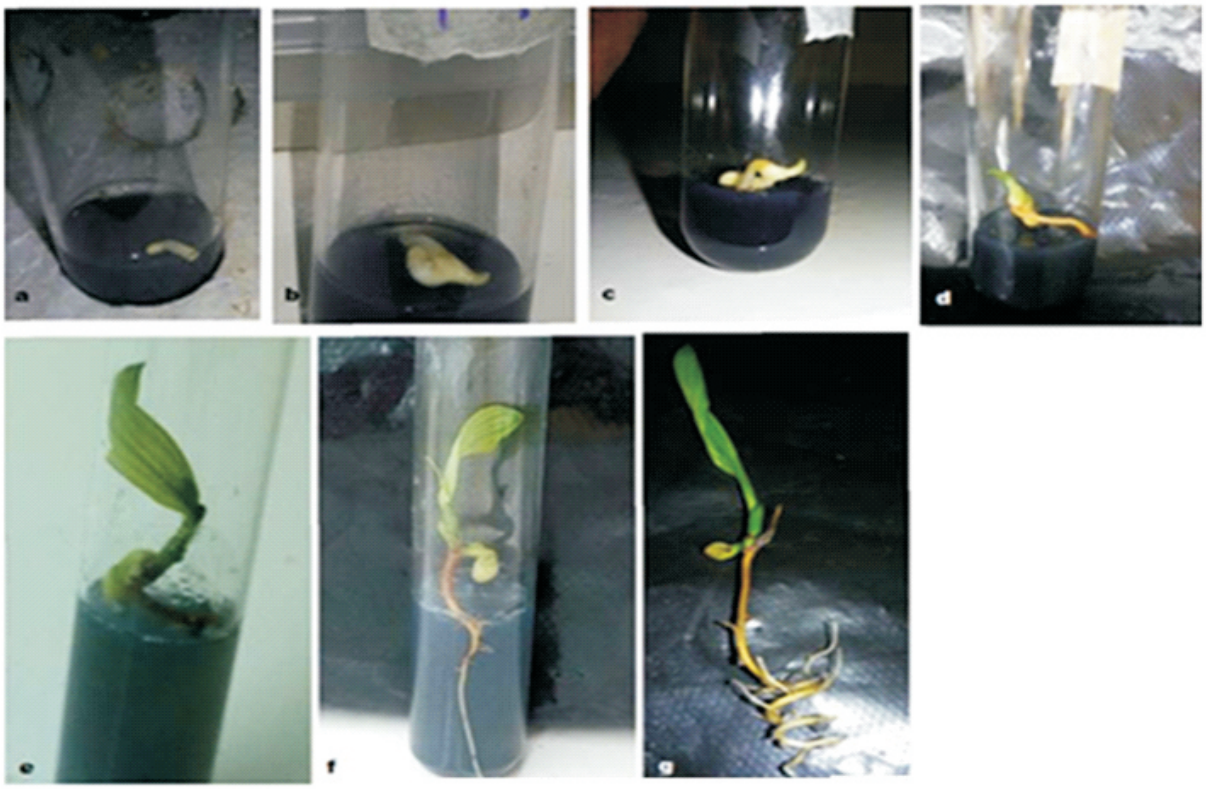

Plate 2: Growth pattern of zygotic embryo of *Elaeis guinensis* cultured on Eeuwens medium

a. A culture of zygotic embryo **b**: Swelling of zygotic embryo, 10 days after inoculation **c**: Formation of haustorium and colour change of embryonic axis **d**: Formation of shoots and roots 3 weeks after inoculation; **e**: Shoots and roots from zygotic embryo 6 weeks after inoculation **f**: Plantlet of *Elaeis guinensis* 8 weeks after inoculation **g**: Plantlet of *Elaeis guinensis* 10 weeks after inoculation

increased progressively as the culture was maintained over the period of time. They increased vigorously from week 4 to week 10. This was in line with the findings of Zati et al. (2014) that fresh weight and length of shoots of *Elaeis guineensis* increased when cultures were maintained for a longer period of time.

The morphogenetic responses in zygotic embryo germination of 'Tenera-albescens' form-variety of oil palm cultured in Eeuwens medium supplemented with varying concentrations of NAA and BAP alone and in combinations followed direct organogenesis pathway to regenerate plantlets. Plantlet of date palm was similarly obtained by direct organogenesis using NAA, IAA, BAP and 2, 1-P in Murashige and Skoog's medium (Asemota and Eke, 2005). The present study also demonstrated that zygotic embryo germination of 'albescens' type of 'tenera' of oil palm in Eeuwens medium was enhanced by plant growth regulators both alone and in combinations with significant differences occurring among treatments. Each of the growth regulator treatment promoted zygotic embryo germination and seedling emergence.

The control which had no growth regulator could not support embryo germination.

This was similar to report of Shittu (2003) who also observed no response in oil palm embryos cultured in Eeuwens medium without growth regulator. However, Thawaro and Te-chato (2010) reported that seedlings required only the basal medium without plant growth regulator, and this differs from the findings of the current study. They posited that the embryo is capable of synthesizing plant regulator itself. Although their work was done with MS medium, the current study was with Eeuwens medium, which may not have supported the embryos in synthesizing plant regulator *in situ*. Rillo and Palomo (1990) and Areza-Ubaldo et al. (2003) reported that coconut embryos grew best in Eeuwens medium without plant growth regulators. The

Table 2: Effect of NAA and BAP media supplementation on shoot emergence and shoot length of 'Albescens' embryos cultured in Eeuwens medium

Growth regulators		Shoot emergence (days)	Shoot length (cm) 3 WAI
NAA (mg/L)	BAP (mg/L)		
0 .00	0.00	NR	NR
0.05		14.75^{a}	0.47^{c}
0.10		14.38^{a}	0.58^{bc}
0 .00	0.05	14.38^{a}	0.45^{c}
0.05		14.38^{a}	0.54^{bc}
0.10		14.38^{a}	0.66^{b}
0.0	0.10	14.75^{a}	0.53^{bc}
0.05		14.18^{a}	0.64^{b}
0.10		14.18^{a}	0.91^{a}
SED		0.33	0.05

^{abc}= Means in the same column with different superscript differ significantly (P<0.05), NR = No response; WAI = weeks after inoculation; NAA = 1-Naphthalene Acetic Acid; BAP = 6-Benzylaminopurine; SED = Standard error difference of means

Table 3: Mean values of days to root emergence and root length of 'Albescens' embryos treated with growth regulators

Growth regulators		Root emergence (days)	Root length (cm)			
NAA mg/L	BAP mg/L		4 WAI	6 WAI	8 WAI	10 WAI
0 .00	0.00	NR	NR	NR	NR	NR
0.05		17.62^{ab}	0.51^{cd}	1.47^{e}	2.39^{e}	4.36^{f}
0.10		17.75^{a}	0.52^{cd}	1.55^{de}	2.58^{d}	4.63^{e}
0 .00	0.05	15.56^{cd}	0.48^{d}	1.48^{e}	2.28^{e}	4.40^{f}
0.05		15.19^{cd}	0.53^{cd}	1.59^{d}	2.78^{c}	5.01^{c}
0.10		15.19^{cd}	0.58^{c}	1.78^{c}	2.88^{c}	4.83^{d}
0.0	0.10	16.56^{ac}	0.53^{cd}	1.51^{de}	2.44^{de}	4.41^{f}
0.05		15.31^{cd}	0.71^{b}	2.04^{b}	4.18^{b}	6.45^{b}
0.10		15.31^{cd}	0.82^{a}	2.48^{a}	4.98^{a}	6.91^{a}
SED		0.52	0.03	0.04	0.08	0.06

difference in result probably could be due to the difference in species of plants used as the current study was on oil palm embryos.

The analysis of variance of data obtained showed that there were no significant differences among the treatment concentrations of plant growth regulators on days to shoot emergence. But significant differences ($p < 0.05$) on the effect of the growth regulators on root emergence and shoot length were obtained. The treatment combination 0.10 NAA + 0.10 BAP, had the earliest mean value for shoot emergence 14.18 days (Table 2) which was not significantly different from the other plant growth regulators applied alone and in combination. Similar result was reported by Bhattacharya et al. (2003) on papaya *in vitro* regeneration where NAA and BAP had a combined effect on shoot initiation from mature embryo. The treatment combination, 0.10 NAA + 0.10 BAP also had the highest shoot

length 0.91cm (Table 2) and was significantly different ($p < 0.05$) from the shoot length in the other treatment concentrations of the plant regulators. Root emergence was also earlier in the combined growth regulator treatments than when applied alone (Table 3).

The least mean values for root length were also obtained in 0.00 NAA + 0.05 BAP (Table 3) at 4, 6, 8, and 10 WAI of 0.48 cm, 1.48 cm, 2.28 cm and 4.40 cm respectively. Results obtained showed that 0.05 NAA + 0.00 BAP at 4, 6, 8 and 10 WAI had root lengths of 0.51 cm, 1.47 cm, 2.58 cm and 4.36 cm respectively. However, the results generally showed that plant growth regulators in combination performed better than when alone. This supports Suranthran et al. (2011) that the synergy between NAA and BAP exhibited positive effect on the *in vitro* propagation of oil palm. Najya et al. (2013) also observed that combination of NAA, BAP and activated charcoal provided more root system and shoot growth in zygotic embryos of dwarf coconut. It seems that low concentrations of auxin and cytokinin combination are suitable for the establishment of oil palm zygotic embryo. The growth inducing effect of NAA with BAP was also reported by Gantait et al., (2010), where MS medium fortified with a low level of NAA and BAP promoted earliest bud initiation in *Gerbera jamesonii*. The synergistic effect of BAP and NAA in the establishment of zygotic embryo and its development during this study was evident. Samarina et al. (2010) also reported similar incident in a study in lemon micropropagation. They found that the combining effect of low concentrations of NAA, BAP and GA_3 (gibberellic acid) played decisive role in the enhanced morphogenesis in all four lemon cultivars studied. Nevertheless, it can be assumed that both NAA and BAP basically enhanced the shoot and root initiation

Plant height, root length, number of leaves and number of roots varied significantly ($p < 0.05$) in response to plant growth regulators. The highest root lengths of 0.82cm, 2.48 cm, 4.98 cm, and 6.91 cm (Table 3) at 4, 6, 8 and 10 WAI respectively, plant heights of 2.21 cm, 7.42 cm, 10.42 cm and 13.96 cm (Table 4), and mean number of leaves 3.13 and mean number of roots 9.75 at 10 weeks after inoculation (Table 5), were obtained in 'Albescens' embryos grown in Euweens medium supplemented with 0.10 NAA + 0.10 BAP. This was significantly ($p < 0.05$) different from the other concentrations of growth regulators alone and in combinations. Suranthran et al. (2011) had also reported that plant growth regulators had significant ($p < 0.05$) influence on plantlet height, root length and stem diameter of *Elaeis guineensis* Jacq. The concentration 0.1 mg/L of the plant growth regulators gave best results in growth and development of zygotic embryos of the oil palm genotype studied. This was followed by treatment combination of 0.05 NAA + 0.10 BAP at 4, 6, 8 and 10 WAI resulting in plant heights of 1.92 cm, 5.91 cm, 8.63 cm, and 11.81 cm

Table 4: Mean values for plant height of plantlets of 'Albescens' embryos cultured in Eeuwens medium supplemented with growth regulators

Growth regulators		Plant height (cm)			
NAA mg/L	**BAP mg/L**	**4 WAI**	**6 WAI**	**8 WAI**	**10 WAI**
0.00	0.00	NR	NR	NR	NR
0.05		1.52^{e}	4.20^{g}	5.61^{g}	7.88^{f}
0.10		1.64^{d}	4.61^{e}	6.69^{f}	9.25^{e}
0.00	0.05	1.48^{e}	3.91^{h}	5.49^{g}	7.76^{f}
0.05		1.64^{d}	5.04^{d}	7.48^{d}	10.58^{d}
0.10		1.76^{c}	5.36^{c}	8.00^{c}	11.11^{c}
0.00	0.10	1.57^{de}	4.44^{f}	6.94^{e}	9.24^{e}
0.05		1.92^{b}	5.91^{b}	8.63^{b}	11.81^{b}
0.10		2.21^{a}	7.42^{a}	10.45^{a}	13.96^{a}
SED		0.04	0.08	0.11	0.07

^{abc}= Means in the same column with different superscript differ significantly ($P<0.05$); NR = No response; WAI = weeks after inoculation; NAA = 1-Naphthalene Acetic Acid; BAP = 6-Benzylaminopurine, SED = Standard error difference of means

Table 5: Mean values of number of leaves and roots of 'Albescens' plantlets cultured in Eeuwens medium supplemented with growth regulators

Growth regulators		Number of leaves (10 WAI)	Number of roots (10 WAI)
NAA mg/L	BAP mg/L		
0 .00	0.00	NR	NR
0.05		1.94^{c}	1.00^{e}
0.10		2.00^{c}	1.00$_{e}$
0.00	0.05	2.00^{c}	1.00^{e}
0.05		2.06^{c}	5.06^{d}
0.10		2.44^{b}	6.31^{c}
0.00	0.10	2.06^{c}	1.13^{e}
0.05		2.50^{b}	6.94^{b}
0.10		3.13^{a}	9.75^{a}
SED		0.14	0.27

^{abc}= Means in the same column with different superscript differ significantly $p < 0.05$); NR = No response; WAI = weeks after inoculation; NAA = 1-Naphthalene Acetic Acid; BAP = 6-Benzylaminopurine; SED = Standard error difference of means

The least mean values on plant height were obtained from the treatment 0.00 NAA + 0.05 BAP of 1.48 cm, 3.91 cm, 5.49 cm and 7.76 cm at 4, 6, 8, and 10 WAI respectively which were not significantly ($p > 0.05$) different from those of 0.05 NAA + 0.00 BAP at 4, 8, 6 and 10 WAI and with plant heights of 1.52cm, 4.20 cm 5.61 cm, and 7.88 cm respectively.

Plant height and root length of 'Albescens' oil palm increased progressively as the culture was maintained for a longer period of time (Plate 2e - g). The increase was vigorous from week 4 to week 10. This was in line with the findings of Zati et al., (2014) that fresh weight and number of shoots of *Elaeis guineensis* increased when cultures were maintained for a longer period of time.

Conclusions

The study has demonstrated a preliminary result on the possibility of producing plantlets *in vitro* from the 'tenera-albescens' type form-variety of oil palm embryo using Eeuwens medium supplemented with plant growth regulators. NAA and BAP played essential role in regenerating plantlets as supplements in Eeuwens basal medium especially in combination of 0.10 mg/L NAA + 0.10 mg/L BAP. Micropropagation and large scale seedling production is therefore feasible from the plantlets so regenerated. The finding in this study therefore strengthens mass production of 'Albescens' seedlings through micropropagation. 'Albescens' being an extremely rare fruit-type, with low carotene in the mesocarp, and if produced on a large scale could reduce the cost of bleaching particularly in the industrial use of palm oil. Moreover, the genetic integrity and purity of the genotype could be largely conserved and preserved by *in vitro* propagation technique. The control which had no growth regulator could not support embryo germination. The findings obtained in this study can be used to complement conventional oil palm seedling development and production processes for the oil palm industry, and for the benefit of increasing world population.

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