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Explant Age, Auxin Concentrations and Media Type Affect Callus Production from Oil Palm (*Elaeis Guineensis*) Embryo Axes

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Abstract
The effects of age (15 and 18 weeks after anthesis) of explants from oil palm (Elaeis guineensis) embryo axes, media type (Eeuwens and Murashige & Skoog) supplemented with various concentrations (10, 20, 30, 40, 50, 60 and 120 mgl-1) of 2,4-D on callus production, employing standard in vitro techniques were investigated. The results of the study showed that the type of response, time of initiation of callus, percentage germinating explants or percentage callusing explants were greatly affected by the age of explants, the concentrations of the growth regulator and type of media used. It was found that callus generation was best in the 18 weeks after anthesis embryo axes culture in Eeuwens' medium supplemented with 2,4-D. The optimum concentration of the hormone was found to be 50 mgl^{-1} which initiated callus within 35 days in culture. The percentage callusing explants was 80 %.

Keywords: *in vitro*, callus, explant, auxin, culture, embryo axes

Introduction
In vitro culture has found wide applications in biotechnology for crop improvement (1; 2) and commercial exploitation (3; 4). *In vitro* culture refers to the culture of living materials (explant) on a defined nutrient medium, under sterile conditions (5). Under such conditions, the cells, tissues or organs multiply, continue with unorganised growth, known as callus and differentiate to regenerate whole plants (6). Narayanaswamy in 1975 (7) defined callus as a rapidly proliferating undifferentiated mass of cells arising from an isolated differentiated tissue cultured on a nutrient medium containing specific growth hormones such as auxins and cytokinins. *In vitro* methods can be used to propagate a mother plant with desired characteristics, thus eliminating the problems of conventional propagation. More so, it has also been applied to areas such as somatic hybridization of sexually incompatible genera, genetic engineering and the production of rare and expensive plant products by cells in cultures. Presently, the most frequently used *in vitro* cultures are callus (tissue), cell suspension, organ, anther, pollen and protoplast cultures (8). Such cultures have provided information, which aids the understanding of the physiology of isolated plant parts and their interrelationship with the intact plants.

Different types of explants are used for *in vitro* propagation of perennial crops. These include buds or meristem tips, zygotic embryos, differentiated organs and tissues, pollen and haploid cells of the female gametophyte. Several studies have shown that the growth stage of the explants is a critical factor affecting callus induction and plant regeneration (9, 10 & 11). For example, organogenesis is absent in explants obtained from more matured regions of an explant (12). Growth regulators are important in the induction and maintenance of callus and also in the induction of cell differentiation and morphogenesis. The type and amount of growth regulators in media are other important factors affecting the induction and maintenance of callus and also in the induction of cell differentiation and morphogenesis. The media used for callus induction vary depending on the type of explants (13, 14, 15, 16 & 17). *In vitro* propagation methods have been successfully developed for oil, date, coconut and some ornamental palms (18).

The oil palm, *Elaeis guineensis*, Jacq. is one of the most important tree crops in Nigeria. It is one of the main sources of vegetable oil for food and feeds. Seeds are generally used to propagate oil palm, although, there are some limitations associated with this (19). These have necessitated clonal propagation by tissue culture technique. To generate embryogenic calli capable of regenerating into whole plant, optimal physiological condition of donor plants, determination of the most productive explants, medium composition and the induction conditions must be evaluated for any plant before it's *in vitro* culture (20). The aims and objectives of this study are to investigate the effects of explant age (oil palm embryo axes 15 and 18 weeks after anthesis {WAA}), auxin (2,4-Dichlorophenoxy acetic acid {2,4-D}) concentrations in different media (Murashige & Skoog {MS} and Euewens) on callus production from *E. guineensis* embryo axes, employing standard *in vitro* techniques.

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Materials and Method

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

Plant material used

Seeds of the oil palm (*E. guineensis*) used for this study were obtained from the Seed Production Division (SPD), NIFOR, Benin City. All seeds used were of the Tenera fruit form. The seeds were harvested at different stages after anthesis. Embryo axes from these seeds were carefully extracted and used.

Culture and media preparation

The effect of two types of nutrient media namely MS and Eeuwens' media was investigated. The stock solutions for macro elements (A), micro elements (B), Iron source and organic nutrients of these media were prepared by accurately weighing out the respective salts (21 & 22). Each salt was weighed and dissolved in distilled water separately before mixing, in order to avoid formation of complex insoluble salts. The stock solutions were stored at 4 ^{o}C until required for use. The preparations were melted in an autoclave at 1bs/m^2 pressure for 50 min at 100 ^{o}C and allowed to cool down before use. Following the melting of the stock solutions, the complete media was prepared as described by Dixon (12). Each of the melted media containing the various concentrations (10, 20, 30, 40, 50, 60 and 120 mgl^{-1}) of hormones were dispensed into labelled McCartney bottles giving seven (7) replicates for each treatment. These were autoclaved at 151 bs/m^2 pressure for 20 min at 121 ^{o}C. After sterilization, the McCartney bottles were shaken gently to uniformly disperse the charcoal in the medium in the bottles. They were allowed to cool and solidify at room temperature.

Extraction, surface sterilization and inoculation of embryo axes

Viability test was first carried out on the seeds. This is useful in determining the wholesomeness of the seeds to be used. The test employed in this study was the floatation method. The seeds were placed in distilled water and those that floated were regarded as non-viable. The viable seeds were then soaked in distilled water for two hours to soften the endocarp tissue. The embryo axes were extracted using a sterilized scapel and a pair of forceps. The extracted embryos were surface sterilized by rinsing in 21 % sodium hypochlorite for 5 minutes. Traces of the sodium hypochlorite were removed by rinsing three times with distilled water (total rinse time 1h 30 min) to get rid of the sterilizing solution, which is toxic to the explants at prolonged exposure. The embryos were then transferred into McCartney bottles containing the media using sterilized forceps. The cultures were incubated in a Gallenhamp incubator at 28 ^{o}C in the dark.

Statistical analysis of data

Each treatment was replicated 7 times and results represent mean $\pm$ standard deviation. The statistical tools used in this study include one and two way analysis of variance (ANOVA),
Duncan's New Multiple Range (DMR) and Student T- Test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 14.

Results

The results obtained when 15 and 18 WAA embryo axes were cultured in MS medium supplemented with 2,4–D are represented in Table 1. In the 15 WAA embryo axes culture, germination was only obtained at 10, 20 and 30 mgl^{-1} 2,4–D treatments within 28, 32 and 45 days in culture respectively. Callus was only produced at 40 mgl^{-1} 2,4-D treatment within 42 days in culture. The 18 WAA embryo axes culture favoured callus production more than the 15 WAA embryo axes culture. Although the embryos germinated in 10 and 20 mgl^{-1} 2,4–D treatment within 32 and 35 days respectively, callus was produced in 30 – 120 mgl^{-1} 2,4-D treatment within 35 and 46 days in culture. Thus, the 18 WAA embryo axes favoured callus production more than the 15 WAA embryo axes.

Table 1: 2,4-Dichlorophenoxy acetic acid concentration in MS medium and embryo age affect time of initiation and type of response in oil palm embryo axes culture

2,4-D	Age of zygotic embryo			
concentration	15 weeks after anthesis		18 weeks after anthesis	
mgl^{-1}	Time (days)	Type of response	Time (days)	Type of response
0	NR	NR	NR	NR
10	28.0 $\pm$ 1.7^{a}	germination	32.0 $\pm$ 1.4^{a}	germination
20	32.0 $\pm$ 2.1^{b}	germination	35.0 $\pm$ 1.1^{a}	germination
30	45.5 $\pm$ 2.1^{c}	germination	42.0 $\pm$ 2.1^{b}	callus
40	42.0 $\pm$ 1.9^{c}	callus	42.0 $\pm$ 2.4^{b}	callus
50	NR	NR	38.0 $\pm$ 1.2^{a}	callus
60	NR	NR	45.0 $\pm$ 1.9^{b}	callus
120	NR	NR	46.0 $\pm$ 2.3^{b}	callus

Legend: Values are means of seven replicates ± standard deviation. Means with similar alphabet in each vertical column are not significantly different (P=0.05). MS= Murashige and Skoog's; NR= no response; germination= initiation of germination; callus= callus formation.

Table 2 shows the time of germination or initiation of callus in 15 and 18 WAA embryo axes cultured in Eeuwens' medium supplemented with 2,4-D. In the 15 WAA embryo axes culture, 2,4-D suppressed germination, while it favoured callus production at 40-120 mgl^{-1} within 45–49 days in culture. Similarly in the 18 WAA embryo axes culture, callus was produced from all the treatments (10 - 120 mgl^{-1} 2,4-D) within 35 – 46 days in culture. Callus was produced at the shortest time in 50 mgl^{-1} 2,4-D treatment in 35 days.

Table 2: 2,4-Dichlorophenoxy acetic acid concentration in Euewens' medium and embryo age affect time of initiation and type of response in oil palm embryo axes culture

2,4-D	Age of zygotic embryo			
concentration	15 weeks after anthesis		18 weeks after anthesis	
mgl^{-1}	Time (days)	Type of response	Time (days)	Type of response
0	NR	NR	NR	NR
10	NR	NR	46.0 ± 3.2^{a}	callus
20	NR	NR	42.0 ± 2.6^{a}	callus
30	NR	NR	38.0 ± 0.8^{b}	callus
40	49.0 ± 2.4^{a}	callus	46.0 ± 1.9^{a}	callus
50	45.0 ± 2.1^{a}	callus	35.0 ± 0.7^{c}	callus
60	49.0 ± 1.8^{a}	callus	46.0 ± 2.2^{a}	callus
120	49.0 ± 1.9^{a}	callus	38.0 ± 1.1^{b}	callus

Legend: Values are means of seven replicates ± standard deviation. Means with similar alphabet in each vertical column are not significantly different (P=0.05). NR= no response; germination= initiation of germination; callus= callus formation.

The percentage germination and callusing explants of 15 and 18 WAA embryo axes cultured in MS medium supplemented with 2,4-D are shown in Figure 1. For both the 15 and 18 WAA embryo axes cultures (Figure 1A), the highest response was obtained at 30 and 10 mgl^{-1} 2,4-D treatment respectively for which 40 % of the explants germinated. For callus production (Figure 1B), the only callus initiation was reduced at 40 mgl^{-1} 2,4-D treatment, for which 20 % of the explants responded in the 15 WAA embryo axes culture. For the 18 WAA embryo axes culture, the highest callus production were obtained at 50 and 120 mgl^{-1} 2,4-D treatment, for which 60 % of the explants responded.

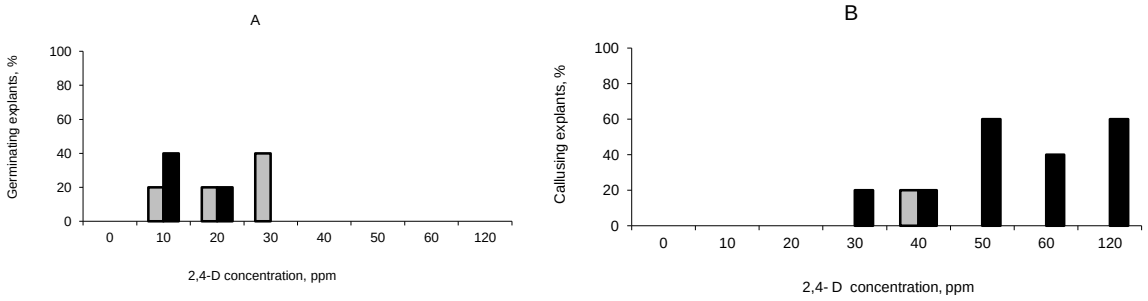

Figure 1: 2,4-Dichlorophenoxy acetic acid concentration in Murashige and Skoog's medium determines initiation of germination or callusing explants of 15 weeks (grey bar) and 18 weeks (dark bar) after anthesis oil palm embryo axes culture

The percentage germinating and callusing explants of 15 and 18 WAA embryo axes of *E. guineensis*, cultured in Eeuwens' medium supplemented with 2,4-D are shown in Figure 2. For percentage germination, 20 % of the explants germinated only at 0 mgl^{-1} 2,4-D treatment in the 18 WAA embryo axes culture (Figure 2A). Callus production was observed at 50 mgl^{-1} 2,4-D treatment for which 40 % of the embryo axes produced callus in the 15 WAA embryo axes culture (Figure 2B). Other treatments initiated callus in 20 % of the explants in Eeuwens' medium supplemented with 2,4-D treatment. The best result for callus production was observed in 18 WAA embryo axes culture. The highest callus production was obtained at 50 mgl^{-1} 2,4-D treatment, for which 80 % of the explants produced callus. Other treatments initiated callus in 40 – 60 % of the explants.

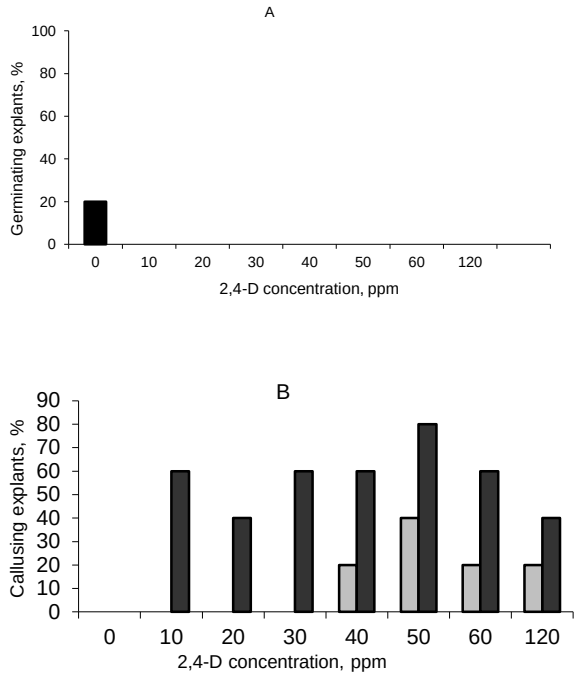

Figure 2: 2,4-Dichlorophenoxy acetic acid concentration in Euewens' medium determines initiation of germination or callusing explants of 15 weeks (grey bar) and 18 weeks (dark bar) after anthesis oil palm embryo axes culture

Discussion

The choice of explants with the right properties is critical in in vitro techniques. The variation in regenerative behaviour among explants is sometimes attributable to the age of the tissue or organ and the extent to which the constituent cells are differentiated. Callus initiation and embryogenesis are dependent on age of explants. Young soft (non woody) tissues respond better in culture than older woody tissues (5). Various portions of the same explants also respond differently in culture. In the case of specialized tissues such as endosperm, callus proliferation may be highly dependent upon the time of excision. In this study, a preliminary experiment that was initially conducted revealed that 12 WAA oil palm embryo axes were not suitable for callus induction, as they were not fully formed in the nut. The 18 WAA embryo axes are slightly bigger than the 15 WAA embryo axes. During the course of the research, across Tables 1-2 and Figures 1-2, it was observed that explant age affects callus production as 18 WAA embryo axes were more suitable than 15 WAA embryo axes. For example, Tables 1 and 2 show the time of initiation of response for 15 and 18 WAA oil palm embryo axes culture in either MS or Eeuwens' medium supplemented with varying concentrations of 2,4-D. Callus initiation was observed as early as 35 days in the 18 WAA embryo axes culture in Eeuwens' medium supplemented with 50 mgl^{-1} 2,4-D (Table 2). In the 15 WAA embryo axes culture, callus was induced in MS medium supplemented with 40 mgl^{-1} 2,4-D after 42 days. Similarly, all concentrations (10-120 mgl^{-1}) supported callus initiation in the 18 WAA embryo axes in Eeuwen's medium, while concentrations from 40-120 mgl^{-1} 2,4-D supported callus initiation in the 15 WAA embryo axes culture (Table 1). The percentage responses for both the 15 and 18 WAA embryo axes in either MS or Eeuwens' medium supplemented with 2,4-D (Figures 1 and 2) show that in all cases, treatments involving 18 WAA embryo axes culture gave higher responses in all cases than the 15 WAA embryo axes. This seems to indicate that the 18 WAA embryo axes favour callus production in E. guineensis compared with the 15 WAA embryo axes. This could be taken to mean that other factors affect callus production apart from concentrations of the auxin, 2,4-D. Dixon (1985) is of the opinion that a great number of factors affect callus induction in in vitro culture experiments. These, according to him, include the physiological conditions of the explants. The source, the age of the explants, size of the explants, the genotype and nature of explants have been reported as factors. In both the 15 and 18 WAA embryo axes used for this research, all these factors were the same except the age.

From Tables 1-2 and Figures 1-2, embryo axes cultured in Eeuwens' medium produced a better result compared with results obtained from MS medium in terms of time of initiation of callus and percentage callusing of explants. It could be inferred that Eeuwens' medium contains a more suitable mineral nutrients mix that are readily available to the developing embryos. Reduced nitrogen in the form of ammonium when used to

supplement the nitrate in the media as in the Eeuwens' medium is reported to be stimulatory to the production of callus (23).

The induction of callus and formation of roots and shoots in plant tissue cultures also appear to be regulated basically by balances of two hormonal substances, auxins and cytokinins as proposed by (24). Other substances seem to modify the effectiveness of the growth regulators. The right amount of auxin is required for specific response. The concept that auxin/cytokinin interaction underlies the process of organogenesis apparently applies to all plants and the inability to extend it to some plants indicates simply a limitation in accessory factors. The concentration of growth media affects the particular response obtained in oil palm embryo axes culture. The results from Table 1-2 show similar trend of response. Low concentrations of auxins favour embryo germination. For callus formation, higher auxin levels were required. For example, in Table 1, embryo axes germinated at concentrations equal to or below 30 and 20 mgl^{-1} of 2,4-D in both the 15 and 18 WAA embryo axes respectively. Callus initiation increased as concentration of 2,4-D increased above the indicated concentrations. In the 18 WAA embryo axes, the best concentration of 2,4-D that gave the best results for the time of initiation of callus and percentage response for callus production is between 40–50 mgl^{-1}. Similarly, in the 18 WAA embryo axes culture, it was 50 mgl^{-1} auxins. It may therefore be deduced that the optimum concentration of auxin for the 15 WAA embryo axes is between 40–50 mgl^{-1} while in the 18 WAA embryo axes it is 50 mgl^{-1} of 2,4-D. This seems to indicate that, callus induction is promoted on media with high level of auxins without cytokinin. It may therefore be deduced that not only the presence of growth regulators, but also the concentration play important roles in the formation and time of callus initiation in oil palm. Those with adequate amounts of the growth regulators produced callus before those without the optimum concentrations. This statement agrees with the report of Dixon (12) and Odewale (25) that growth regulators are important in the induction and maintenance of callus.

In conclusion, the study revealed that the age of explant, concentrations of growth hormones and media type have significant effects on callus initiation from Elaeis guineensis embryo axes using in vitro technique. The results showed that 18 WAA embryo axes are better for callus induction than 15 WAA embryo axes. Preliminary experiment also revealed that 12 WAA embryo axes are absolutely not suitable. MS and Eeuwens' media supported callus production, but by way of comparison, Eeuwens' medium is to be preferred to the Murashige and Skoog's medium. Regarding concentration of growth hormones, the study showed that 50 mgl^{-1} is optimum for this purpose.

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References

1. Quak F: Meristem culture and virus–free plants cell tissue and organ culture. *Crop Science* 19: 340 – 352. 1977.
2. Jaiwal PK and Gulati A: Current status and future strategies of *in vitro* culture techniques for genetic improvement of mungbean (*Vigna radiatea* (L.). *Wikzek Euphytica* 86: 167 – 181. 1995.
3. Muthukumar B, Mariamma H and Gnaman A: Regeneration of plants from primary leaves of cowpea (*Vigna inguiculata* L.). *Plant Cell Tissue and Organ Culture* 42: 152 – 155. 1995.
4. Rajasekaran K: Regeneration of plants from cryopreserved embryogenic cell suspension and callus cultures of cotton (*Gossypium hirsutum* L.). *Plant Cell Report* 15: 859 – 864. 1996.
5. Pierik RIM: *In vitro* culture of higher plants: International course on applied plant breeding. International Agricultural Centre, Wageningen, The Netherlands 107 p 1984.
6. Bhojwani SS and Razdan MK: Plant Tissue Culture: Theory and Practice. Revised Edition. Elsevier Science Publishing Company, New York, 767 p. 1996.
7. Narayanaswamy S: Regeneration of plant from tissue culture. In: Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Reintert, C and Bajaj, YPS (Eds.). Springer Verlag, Berlin, Heidelberg, New York. pp 179 – 248. 1975.
8. George EF, Hall MA and De Klerk GJ: Plant Propagation by Tissue Culture. 3rd Edition, Springer, Dordrecht 501 p. 2008.
9. Ahlowalia BJ: Forage Grasses. In: Handbook of Plant Cell Culture. Ammirato, PVJ, Evans, DA, Sharp, WR, Yamada, Y (Eds). Mc Millian Pub Comp, New York, London, pp 91 - 125. 1984.
10. Botti C and Vasil IK: Ontogeny of somatic embryos of *Pennisetum americanuin* in cultured immature inflorescences. *Canadian Journal of Botany* 62: 1629 - 1635. 1984.
11. Boyes CJ and Vasıl IK: Plant regeneration by somatic embryogenesis from cultured young inflorescenses of *Sorghum arundinaceum* (Desv) stapf var Sudanense (sudan grass). *Plant Sci*ence *Letter* 35: 153 - 157. 1984.

12. Dixon RA: Plant Cell Culture: A Practical Approach. IRL Press Ltd., Oxford, 236 p. 1985.
13. Al-Khayri JM: Date palm *Phoenix dactylifera* L. In: Protocol For Somatic Embryogenesis in Woody Plants. Jain SM, Gupta PK (Eds). Springer, Dordrecht, pp 309 – 319. 2005.
14. Al-Khayri JM and Al-Bahrany AM: Genotype-dependent *in vitro* response of date Palm (*Phoenix dactylifera* L.) cultivars to silver nitrate. *Science Horticulture* 99: 153 –162. 2004.
15. Fki L, Masmoudi R, Drira N and Rival A: An optimised protocol for plant regeneration from embryogenic suspension cultures of date palm, *Phoenix dactylifera* L. cv Deglet Nour. *Plant Cell Report* 21: 517 – 524. 2003.
16. Othmani A, Bayoudh C and Drira N: Somatic embryogensis and plant regeneration in date palm, *Phoenix dactylifera* L., cv Boufeggous is significantly improved by fine chopping and partial desiccation of embryogenic callus. *Plant Cell Tissue Organ Culture* 97:71 – 79. 2009.
17. Sané D, Aberlenc-Bertossi F, Gassama-Dia YK: Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera*). *Annal of Botany* 98: 301 – 308. 2006.
18. Blake J: Tissue culture propagation of coconut, date and oil palm. In: Tissue Culture of Trees. Dodds, JN (Ed). Room – Helm Ltd., Beckenham, Kent, U.K, pp 29 – 50. 1983.
19. Shittu HO: Callus generation from oil palm (*Elaeis guineensis*, jacq.) using embryo explants. M.Sc. Thesis. University of Benin, Benin City, 74 p. 2003.
20. Lörz H, Gobel E, Brown P: Advances in tissue culture and progress towards genetic transformation of cereals. *Plant Breeding* 100: 1 - 25. 1988.
21. Eeuwens CJ: Mineral requirement for growth and callus initiation of tissue explants excised from mature palms (*Cocos nucifera*) and cultured *in vitro*. *Physiologia Plantarium*, 36: 23 – 28. 1976.
22. Murashige T and Skoog F: A realized medium for rapid growth and bioassays with tobacco cultures. *Plant Physiology* 15: 473 – 497. 1962.
23. Yatazawa M and Futuhashi K: Nitrogen sources for the growth of rice callus tissue. *Soil Science and Plant Nutrition* 14: 73-79. 1969.
24. Skoog F and Miller CO: Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. Symposium of the Society for Experimental Biology, London, pp 118 –131. 1957.
25. Odewale JO, Eke CR, Sogeke AK and Enonuya DOM: Varietal response of oil palm leaf explant to various Naphthalene Acetic Acid (NAA) leaves in Eeuwens tissue culture medium. *The Nigerian Journal of Genetics* 2: 76 – 79. 1996.