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RAPID CALLUS PROLIFERATION, SOMATIC EMBRYOGENESIS AND ORGANOGENESIS OF OIL PALM (*Elaeis guineensis* Jacq.)

Keywords: *Elaeis guineensis* Jacq., Eeuwens (1976) medium, Somatic embryogenesis, NAA, Kinetin, Leaf explant.

SOGEKE, A K*

Modified Eeuwens (1976) medium containing 5mg/l NAA (Y3 (3A)) promoted the fast growth of callus tissues of Elaeis guineensis *Jacq. at the first passage. Subsequent passages of the cultures on Y3(3A) produced callus nodules, green embryoid nodules and few plantlets but callus nodules were predominant. Spontaneous somatic embryogenesis of the callus tissues was stimulated at the first passage on DM(1) which was the modified Eeuwens (1976) basic medium (Y3(O)) supplemented with NAA (0.5mg/l) and Kinetin (2.0mg/l). Embryoids were also obtained from callus on the initiation medium (Y3(3)) and the maintenance medium (Y3(3A)). Subsequent passages on DM(1) resulted in the formation of more green embryoid nodules, leaf primordia, well developed leaves, roots and plantlets. Furthermore, the transfer of the embryogenic tissues from Y3(3A) and DM(1) media to Y3(0) enhanced the production of more plantlets. Also, the formation of adventitious roots by shoots and plantlets was stimulated on the Murashige and Skoog medium (1962) as modified by Hyndman* et al. *(1982). Pneumathode-like structures were occassionally observed in the callus cultures particularly on the explant with callus. The plantlets have subsequently been planted in the field where they are growing satisfactorily.*

* Nigerian Institute For Oil Palm Research (NIFOR), P.M.B. 1030, Benin City, Nigeria.

INTRODUCTION

The totipotency, that is, the capacity of differentiated plant cells to retain their full genetic potential and express them under appropriate conditions, is well known in the plant kingdom and it may be right to claim that all healthy plant cells are totipotent (Ronchi, 1991). However, the capacity of regeneration *in vitro* (organogenesis and particularly somatic embryogenesis) is a property of some genotypes of particular species, often from tissues of specific reproductive organs (*e.g.* pollen, anther, ovule) and induced by stress treatments either chemical (hormonal or organic compounds) or physical (temperature changes, light, wounding) (Rabechault and Martin, 1976; Husemann and Reinert, 1976 and Ronchi, 1991). However, the acquisition of competence to differentiate as a shoot or an embryo appears to be limited to selected cells which due to their spatial location or time of expression, may start a specific developmental pathway under appropriate stimuli and/or environmental condition (Ronchi, 1991). Hence the recalcictrance of the expression of totipotency by some plants may be due to the lack of knowledge of the appropriate conditions required for these plant species.

Most of the researchers who have successfully carried out *in vitro* vegetative propagation of the oil palm have used the process of somatic embryogenesis on callusses obtained on 2, 4-D medium (Jones, 1974; Rabechault and Martin, 1976; Paranjothy and Othman, 1982; Nwankwo and Krikorian, 1983; Blake, 1983; Duval *et al.,* 1988 and Paranjothy, 1989). It is generally accepted that using callus tissues involves fairly significant risks of variability between the plantlets obtained and the mother plant (Reisch, 1983; Duval *et al.*, 1988). These risks have always been a cause of concern for researchers working on oil palm vegetative propagation (Smith and Jones, 1970; Noiret, 1981).

More recently abnormalities in the reproductive organs and the frequency of their appearance in vegetatively propagated oil palm have been attributed to certain stages of the *in vitro* culture irrespective of the type of auxin in the medium, (Corley *et al.,* 1986). The clonal plantlets obtained by Rabechault and Martin (1976) required the use of highly dedifferentiated tissue (callus) with a high growth rate which was called fast growing culture (FGC). Embryoids were later obtained from the culture. Mass propagation of plantlets was carried out through the multiplication of tissues at the FGC stage on media rich in chlorinated auxins (Duval *et al.,* 1988). Another process was later developed by Pannetier *et al.* (1981) whereby embryoids were obtained from a small number of embryogenic structures appearing on the callusses which were formerly obtained from fragments of young leaves cultured *in vitro* with 2,4-D. Mass production was subsequently ensured by the occurrence of a secondary embryogenesis phenomenon making it possible to establish strains of proliferating embryoids.

However, both methods of Rabechault and Martin (1976) and Pannetier *et al.* (1981) involved the use of 2,4-D, a chlorinated auxin. Cells of plant callus cultures maintained on 2,4-D are particularly prone to genetic variation (George and Sherrington, 1984). This could be due to the effect of 2, 4-D at high concentration causing mitotic spindle abnormality (Bayliss, 1973). Furthermore Duval *et al.* (1988) suggested that the methodological differences concerning the choice of explant, the type of callus used and the different culturing conditions could lead to positive results. Hence in several laboratories, investigations into the hormonal status in abnormal palms are now being pursued (Paranjothy, 1989). Therefore in these studies, NAA which has less adverse effects than 2,4-D has been used in the initiation of callus from leaf explants of the oil palm. The aim of this work was to develop a non 2,4-D medium which promoted the rapid growth of the callus immediately after callus initiation followed by spontaneous somatic embryogenesis at the very earliest period of the callus phase on a medium devoid of chlorinated auxins with the ultimate hope of avoiding the abnormality often associated with clonal oil palms.

MATERIALS AND METHODS

The initiation of callus cultures from the tender leaves of spear of 3-year old oil palm tree (*Elaeis guineensis* Jacq.) was on modified Eeuwens (1976) inorganic supplemented with sucrose 30g/l; inositol 100mg/l; aneurine-HCL 0.5mg/l; niacin 0.5mg/l; pyriodoxine-HCL 0.5mg/l; casamino acid 500mg/l; 1-Naphthyl1-acetic acid (NAA) 15mg/l. Agar was added at 7g/l; while pH was 5.7. This constituted the initiation medium (Y3(3)).

Fast growing culture was obtained by reducing the amount of NAA in the medium from 15mg/l in Y3(3) to 5mg/l in Y3(3A) which was the maintenance medium. Spontaneous somatic embryogenesis was induced on the modified Eeuwens (1976) basic medium (Y3(0) supplemented with NAA (0.5mg/l) and Kinetin (8.0mg/l) (DM); or DM(l) medium which contained NAA (0.5mg/l) and Kinetin (2mg/l), which was the differentiation medium. Y3(0) was used for the enhancement of plantlet formation from cultures on Y3(3A) and DM(l) while for the stimulation of adventitious roots, Murashige and Skoog (1962) medium (M&S) as modified by Hyndman *et al.* (1982) (MSR) containing no phytohormones was used.

There were ten replicates per treatment. 10ml of each medium were placed in (28ml) McCartney bottles or 25ml into (50ml) Erlenmeyer-flasks. Sterilization was at 15 1bs/sq. inch for 15 mins while incubation was at 28°C under continuous fluorescent light of 200 lux for a passage of five weeks. Inoculum size of callus was *ca.* 50mg. Visual observation of growth and development of the inoculum was made weekly with the final assessment of the cultures for growth and development after five weeks.

RESULTS

(A)

Enhancement of callus growth (Cultures on Y3(3) to Y3(3A)). Callus tissues initiated on Y3(3) were subcultured to fresh Y3(3) medium where the growth was moderate *ca.* 20 times the inoculum (50mg) (*Figure 1*). After five weeks (1st passage) on the Y3(3) medium, the callus tissues were transferred to Y3(3A) medium which contained 5mg/l NAA instead of the 15mg/l in the initiation medium. The callusses grew much faster on Y3(3A) with an average increase of *ca.* 2-3 times that of Y3(3) and up to six times the inoculum of 50mg.

Although rapid growth of the callus ocurred on Y3(3A) during the first two passages, however, after the third passage, growth on Y3(3A) was a mixture of hyaline to whitish embryogenic callus nodules (*Figure 2A*) and green embryoid nodules with some leaves (*Figure 2B*). Embryogenic callus being defined as callus producing embryoids and plantlets (Jelaska, 1974). Generally callus tissues were predominant on this medium while pneumathode-like structures (Hartley, 1988; Blake, 1983) which were observed on the explant with callus still occurred in some cultures. These are white, wavy, tender structures with serrated surfaces growing either aerially or downward into the medium (*Figure 2C*). During the fourth and subsequent passages on Y3(3A), plantlets with hyaline callus and embryoid nodules were observed (*Figure 2D*).

(B)

Stimulation of somatic embryogenesis (cultures on Y3(3) medium to DM and DM(1) media).

Callus tissues after the first passage on Y3(3) medium were transferred to DM and

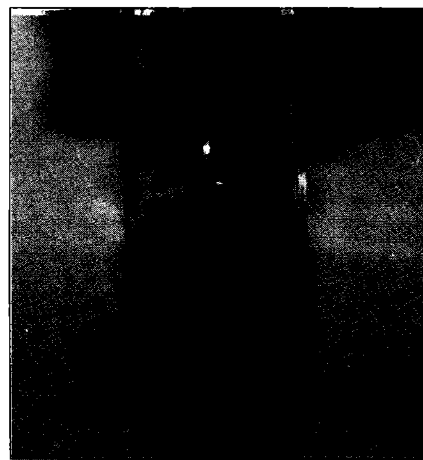

*Figure 1. Whitish callus tissues of oil palm (*Elaeis guineensis *Jacq.) after the first passage of five weeks on the initiation medium (Y3(3)) of modified Eeuwens (1976) containing NAA at 15mg/l as the only phytohormone. Incubation was at 28°C under continuous fluorescent light of 200 lux.*

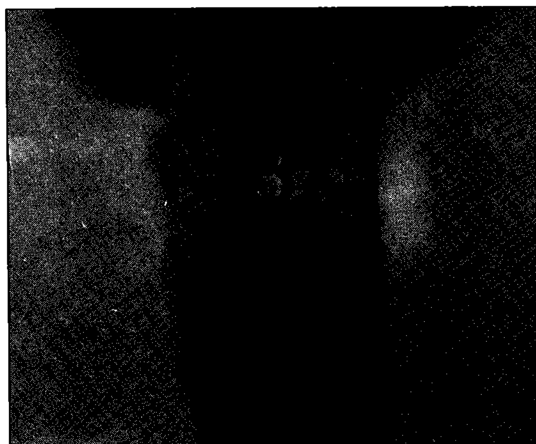

Figure 2A

Figure 2C

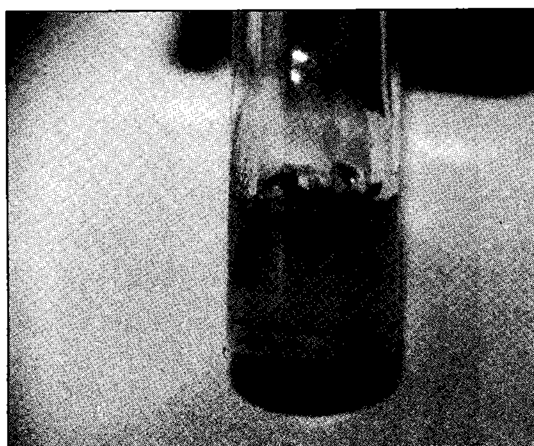

Figure 2B

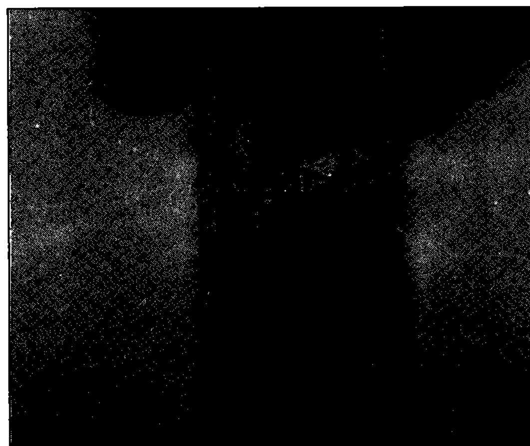

Figure 2D

Figure 2 (A - D). The growth pattern of in vitro *cultures of oil palm (*Elaeis guineensis *Jacq.) on the modified Eeuwens (1976) medium (Y3(3A)) containing NAA (5mg/l) as the only phytohormone. (A) The whitish embryogenic callus. (B) The whitish callus with young leaves. (C) Pneumathode-like structures emanating from the explant with callus on the initiating medium Y3(3). (D) The callus with leaves and root. Incubation was at 28°C under continuous fluorescent light of 200 lux for five weeks.*

DM (1) media for stimulation of somatic embryogenesis. At first passage, spontaneous embryoid nodules occurred on both DM and DM(1) media (*Figure 3A*). The growth pattern on both media varied in between partly hyaline callus and partly green embryoid nodules (*Figure 3B*), shoot leaves only (*Figure 3C*) and callus with root only (*Figure 3D*). There was no marked difference between the cultures on DM and DM(1) media.

However, in order to confirm this observation the following experiments were performed: Cultures on Y3(3A) were transferred to DM and DM(1) separately and also cultures on DM to DM(1) and *vice versa*. Again, there was no marked difference in the type and pattern of the cultures on both media (DM and DM(1)) at the first passage as both stimulated similarly somatic embryogenesis of the cultures from Y3(3A).

Subsequent passages on both DM and DM(1) produced more shoots, plantlets with occasional pneumathode-like structures and callus nodules, roots and also green embryoid nodules. Sometimes callus with several roots were observed. On both DM and DM(1), the stimulation of embryogenesis of the cultures was generally the same (*Figure 4A-F*) no matter the origin of the culture whether from Y3(3) or Y3(3A) at first passage or subsequent passages. However, transfer of cultures from DM or DM(1) to Y3(3A) enhanced callus formation. Since DM(1) contained less amount of Kinetin (2mg/l) than DM(8mg/l) the use of DM(1) as the standard medium for stimulating somatic embryogenesis was therefore adopted.

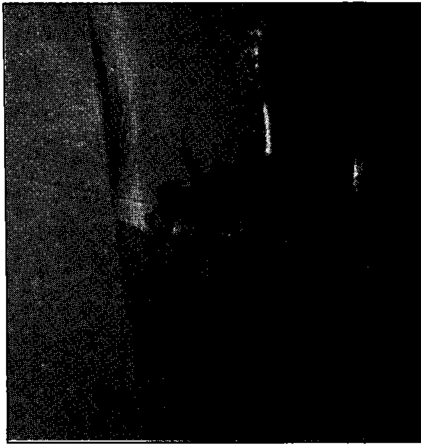

Figure 3A

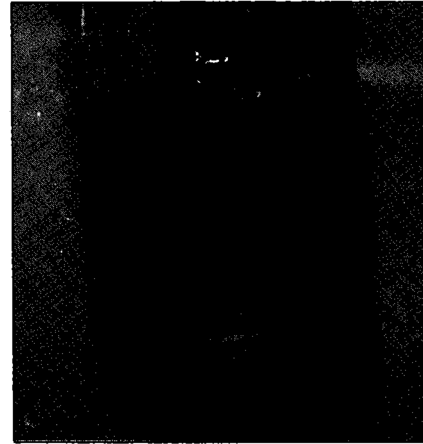

Figure 3B

Figure 3C

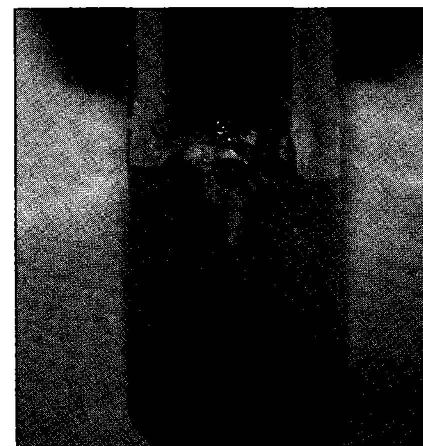

Figure 3D

Figure 3 (A-D). The growth pattern at first passage of in vitro *culture of oil palm (*Elaeis guineensis *Jacq.) on the modified Eeuwens (1976) medium DM (NAA, 05.mg/l and Kinetin, 8mg/l) and DM(1) (NAA 0.5mg/l and Kinetin 2mg/l). (A) Whitish or greenish embryoid nodules. (B) Partly hyaline callus and partly mass of embryoid nodules. (C) Shoot leaves only (D) Callus with root only. Incubation was at 28°C under continuous fluorescent light of 200 lux for five weeks.*

(C)

Enhancement of plantlet formation (cultures on DM(1) and Y3(3A) to Y3(0)).

In order to stimulate the production of more plantlets, callus cultures from DM(1) and Y3(3A) were transferred separately to Y3(0). Both cultures behaved similarly. At first passage, there was moderate growth of the callus with cluster of young leaves, roots, with occasional pneumathode-like structures and some plantlets with primary and secondary leaves. Sometimes there was only little growth of the callus with no organs. Shoots however, were predominant on the Y3(0) with few roots.

At the second passage of the cultures on Y3(0), cluster of shoots with green embryoid nodules were formed. Sometimes leaf primordia supported by roots of 1.5-12mm long were observed. Again shoots were dominant on Y3(0) with few vigorous plantlets (*Figure 5A - C*).

(D)

Stimulation of adventitious roots on MSR medium. On Y3(0), Y3(3A) and DM(1), lateral roots were uncommon. Hence in order to stimulate the formation of adventitious roots, the cultures of shoots or plantlets were transferred to MSR medium.

(a) Y3(0) to MSR:

(i) Plantlets transferred.
Vigorous rooting system with several laterals on each primary root

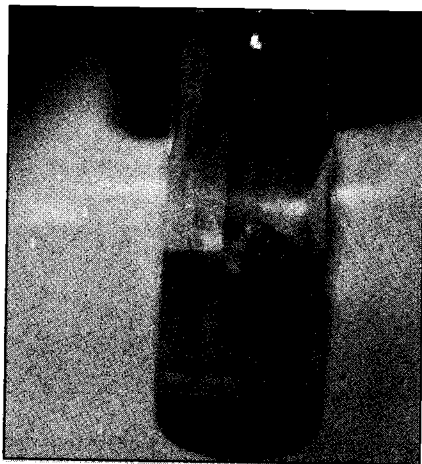

Figure 4A

Figure 4D

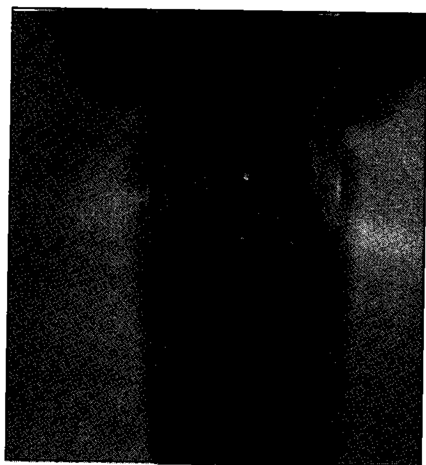

Figure 4B

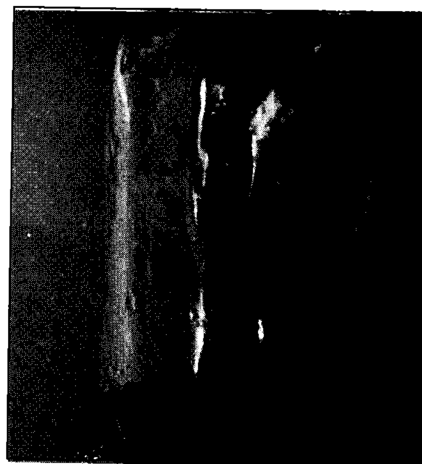

Figure 4E

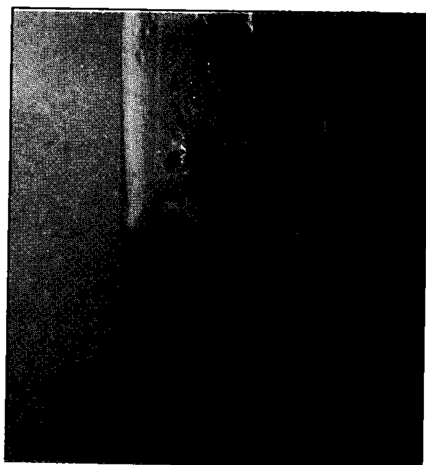

Figure 4C

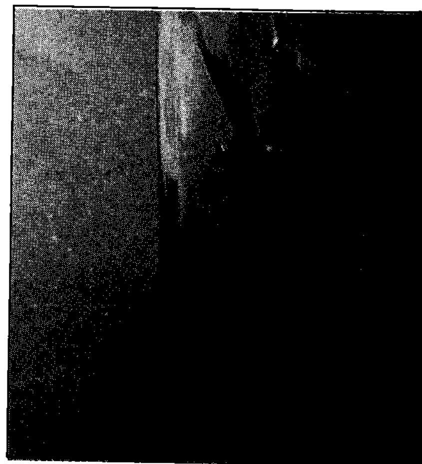

Figure 4F

Figure 4 (A - F). The growth pattern at subsequent passages of in vitro *culture of oil palm (*Elaeis guineensis *Jacq.) on modified Eeuwens (1976) medium DM (NAA, 0.5mg/l and Kinetin, 8mg/l) and DM(1) (NAA, 0.5mg/l and Kinetin, 2mg/l). (A) - Embryogenic callus with shoots and root. (B) The well developed plantlet with emergent roots. (C) Multiple shoots on a callus base with two roots. (D) Plantlets on a callus base with three roots. (E) Twin shoots on the same callus base with root. (F) Triplet shoots on the same callus base with short roots. Incubation was at 28°C under continuous fluorescent light of 200 lux for five weeks.*

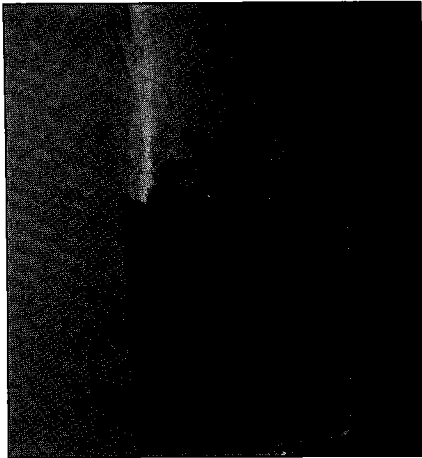

Figure 5A

Figure 5B

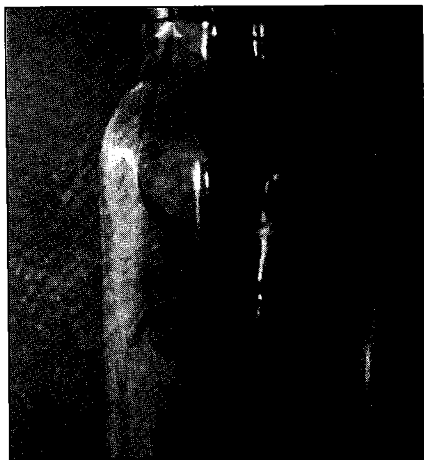

Figure 5C

Figure 5 (A - C). The growth pattern of in vitro *culture of the oil palm* (Elaeis guineensis *Jacq.) on modified Eeuwens (1976) basic medium (Y3(0)). (A) Shoots and embryoids. (B) Shoot leaves only. (C) The well-developed plantlet with short roots. Incubation was at 28°C under continuous fluorescent light of 200 lux for five weeks.*

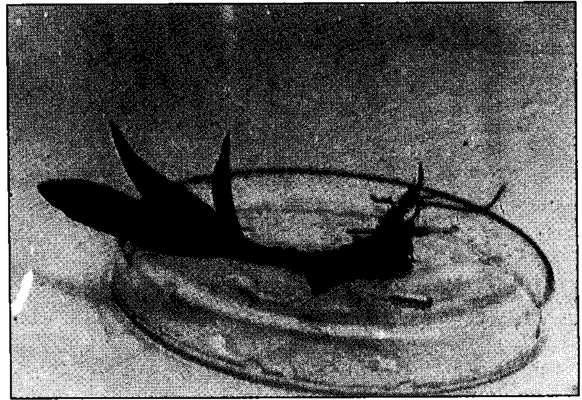

Figure 6A

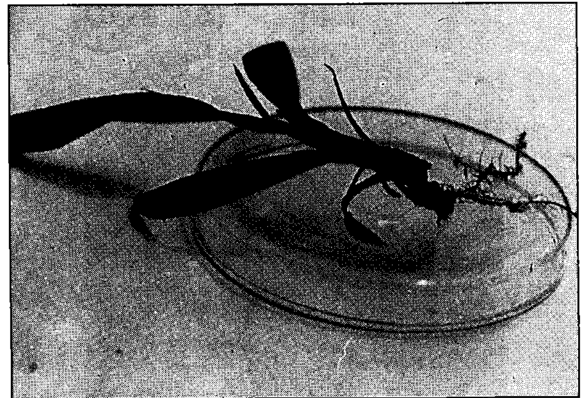

Figure 6B

Figure 6. The well-developed roots of the plantlets of the oil palm (Elaeis guineensis *Jacq.) on Murashige and Skoog (1962) basic medium as modified by Hyndman* et al., *(1982). (A) New primary roots emanating from the plantlet. (B) Primary roots with adventitous roots on the plantlet.*

Figure 7. Roots emanating directly from the oil palm (Elaeis guineensis *Jacq.) leaf explant on the callus initiating medium (Y3(3)) containing NAA at 15mg/l as the only phytohormone after 42 days of incubation at 28°C under continuous fluorescent light of 200 lux.*

(*Figures 6A* and *B*) occurred in 60% of the cultures while 40% still had primary root with no laterals after three passages each of five weeks incubation period.

(ii) Shoots only transferred.
30% formed primary roots with laterals while another 30% had primary roots only and 40% with no root at all.

(iii) Primary roots of plantlets excised.
30% of the cultures formed new roots with laterals; 20% had new primary roots only while 50% did not form any roots after three passages on MSR.

(b) Y3(3A) to MSR:
30% of the shoots (no roots) formed primary roots with laterals, another 30% formed primary roots only, while 40% did not form any root at all.

(c) DM(1) to MSR:
40% of the shoots (no roots) formed primary roots with laterals; 20% formed primary roots only while 40% did not form any root at all.

(E) Further development of the plantlets.

The plantlets which were obtained have passed through the pre-nursery and nursery stages and have finally been planted out in the field where they are growing satisfactorily. The techniques used are similar to those described by other workers (Hartley, 1988) and the palms which started flowering after 28 months in the field have shown no abnormality so far.

DISCUSSION

Callus tissues obtained from oil palm are generally slow growing and require large amounts of 2,4-D for callus initiation (Rabechault *et al.,* 1972; Smith and Thomas, 1973; Jones 1974, Rabechault and Martin, 1976; Pannetier *et al.,* 1981; Hanover and Pannetier, 1982; Blake, 1983). In this study formation of callus by the leaf explants on the initiation medium (Y3(3)) started from 42 to 57 days after inoculation but after the first passage of this callus on Y3(3), the growth averaged *ca.* 20 times the inoculum. However, on transfer of the callusses to Y3(3A) medium which contained less NAA (5mg/l) than Y3(3) (15mg/l), the callus tissues grew much faster, with the average growth increase of *ca.* 2-3 times, than that of Y3(3). This fast growth of the callus provided available material for somatic embryogenesis thereby minimising the duration and the risk of the prolonged callus phase on the vegetative propagation process.

However, it is noteworthy that subsequent maintenance of the callus tissue on Y3(3A) produced hyaline to whitish nodules, green embryoid nodules and some leaves although callus tissues were predominant. Moreover, on the callus initiating medium Y3(3), it was possible to obtain roots directly from the oil palm leaf explant (*Figure 7*). This supports the view of Blake (1983) that the primary medium (Y3(3)) and particularly the auxin (NAA) has a dual role of initiating proliferation of callus cells followed by induction of embryogenesis, that is, the determination of the cells to embryogenesis was already present when the initial explant was taken. However, Blake (1983) did not envisage this for the oil palm where embryogenesis generally occurred after a series of subcultures over several months.

On transfer of the callus tissues of the oil palm after the first passage on Y3(3) and Y3(3A) to DM and DM(1) media, spontaneous somatic embryogenesis occurred at the first passage on the latter media. The spontaniety of somatic embryogenesis on DM and DM(1) at first passage and the development of more embryoids on these media for over one year of subsequent passages further corroborated the view of Blake (1983) that the stimulation of the oil palm callus cells to embryogenesis and organogenesis requires the appropriate concentration of phytohormones in the favourable medium to initiate callus proliferation (NAA 5mg/l in this study) followed

by induction of embryogenesis and organogenesis (NAA 0.5mg/l and Kinetin 2mg/l or 8mg/l). There was, however, no marked difference in the effects of both DM and DM(1) media.

Blake (1983) proposed that for the vegetative propagation of all palms through tissue cutlure, the aim must be to find a medium in which the initial explant forms callus as quickly as possible and allows rapid determination of embryogenic cells so that embryogenic development can occur when transfer is made to the secondary medium. It is hereby claimed that the short duration of 24 to 42 weeks observed in this study from callus initiation on Y3(3) to plantlet on DM and DM(1) coupled with the fast growth of the cultures on Y3(3A), DM and DM(1) media with no decrease in the vigour of the cultures in subsequent passages therefore adequately satisfy these criteria.

Skoong and Miller (1957) reported that the ratio of auxin to cytokinin exerts an influence on the kind of organogenesis (Shoot, root or plantlet) which was formed in the tobacco callus. This finding has since been generally confirmed by later works on other plant species and with other phytohormones (Linsmaier and Skoog, 1965; Rao *et al.,* 1970; Fridborg, 1971; Simonsen and Hildebradt, 1971; Ohyama and Nitsch, 1972; Rao and Harada, 1973; Nadar, *et al.*, 1978; Nwankwo and Krikorian, 1983). The palms appear to follow this basic pattern as confirmed by the present and past results where a high auxin level was required for callus initiation and auxin with cytokinin were used for embryogenesis (Rabechault and Martin, 1976, Evans *et al.,* 1981; Blake, 1983).

The present results are also in line with previous reports (Jones, 1974; Rabechault and Martin, 1976; Nwankwo and Krikorian, 1983; Blake, 1983; Duval *et al.,* 1988; Paranjothy, 1989). Furthermore the enhanced stimulation of the oil palm plantlets by transferring the shoots or embryoids to the basic medium Y3(0) is a common practice in plant cell, tissue and organ culture (Nadar *et al.,* 1978; Nishi *et al.,* 1973). Also transferring the shoots or plantlets to modified M & S medium (MSR) stimulated adventitious roots as previously reported by Hyodman *et al.* (1982) for cultured rose shoots.

Influorescence and bunch abnormalities have been observed in experimental fields raised from seedlings in NIFOR Main-Station (Obasola *et al.,* 1978). Most of these abnormalities were of spontaneous origin while some were artificially induced by spraying leaf bases, axils and apices with α-(2,4,5-Trichlorophenoxy) propionic acid which is a derivative of 2,4-D used routinely for the clonal oil palm. Furthermore, Obasola (1970), 1972) induced parthenocarpic fruits on the oil palm treated with α–(2,4,5-Trichlorophenoxy) propionic acid.

Obasola *et al.* (1978) also found that the production of andromorphic hermaphroditic bunches is very common in *E. guineensis* x *E. oleifera* hybrids and crosses between these hybrids and *E. guineensis.* And that some oil palm progenies produced mantled fruits which have also been observed in the clonal oil palm. These workers further reported that one out of 16 palms (6.2%) derived from seedlings produced hermaphroditic florets in the fields where abnormality was observed.

Furthermore, Hartley (1988) noted that inflorescence abnormalities are by no means uncommon in the oil palm and that the tendency to abnormality must be taken into account by plant breeders. He also wrote that some palms are more disposed to the production of hermaphroditic inflorescence than others and that young palms occasionally produce andromorphic inflorescence. Hence, inflorescence and bunch abnormalities in the oil palm can be classified as spontaneous (natural and of unknown origin); chemically induced (artificial); or genetic (certain hybrids and progenies).

Corley *et al.* (1986) reported that two of their clones which flowered abnormally had not been exposed to 2, 4-D at any stage. This can therefore be attributed to natural causes but it is well known that prolonged callus stage is not advantageous to the clonal oil palm.

Duval *et al.* (1988) found that fast growing callusses obtained on 2, 4-D medium are more

prone to abnormal flowering than the slower growing nodular callus. Butcher *et al.* (1975) reported that there was a greater tendency for their faster growing cultures of *Helianthus annuus* a non-polysomatic species to become polyploid. Furthermore, Shamina (1966) and Sunderland (1973) working with *Haplopappus gracilis* showed that cultures maintained on media containing 2, 4-D had cells with a greater range of ploidy levels than cultures maintained on media with NAA as the auxin. They both similarly concluded that since the 2,4-D cultures had an increase growth rate, 2,4-D may favour the growth of tetraploid cell lines.

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