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REPORT ABOUT TISSUE CULTURE OF *E. guineensis* J.
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INTRODUCTION

The agronomic importance of the *E. guineensis*, the oil palm, is inferred from its high capacity to produce oil, reaching a record of 6 tonnes of oil per hectare (Jones 1983).

The oil palm crop lacks a standard method of propagation such as grafting or by rooted cuttings so that "in vitro" tissue culture represents a unique path of clonal multiplicaton (Jones 1974, Rabéchault & Martin 1976).

On the other hand, there being no clear methodology described in the literature, it has become urgent to know the morphogenetic conditions "in vitro" that would make it possible to more readily achieve clonal propagation of the oil palm. A crucial point in this question is which type of explant to use: whether young leaves or roots.

Considering the limited supply of oil palm material in the Department of Horticulture of Wye College and, the time necessary for observing and evaluating results, it was decided to essay root disinfection procedures, and to test viability of roots from 3 years old seedlings, maintained in pots with unsterilized soil in green house conditions, under natural day length at 25 ± 5°C.

As a parallel and subsidiary activity, embryos were cultured with two purposes: callus induction and germination.

1. DECONTAMINATION PROCEDURE

Seedling palms were removed from their pots and all compost carefully washed from the roots with tap water. Roots were excised and separated into primary roots (brown and/or white) and secondary roots (yellow and/or white). The roots were then decontaminated using the following sequence of treatment: mercuric chloride (Hg C12) or potassium permanganate (K MnO_4), fungicides, activated charcoal, bleach (domestic sodium hypochlorite) and antibiotics, (table 1.) After each of these steps the material was washed with sterilized double distilled water, (DDW).

TABLE 1 - Substances, concentrations and treatment durations used in root disinfection procedure.

Compound	Concentration	Time
Mercuric chloride	1.0 g/l	1.0,2.5 and 5.0 min.
Potassium Permanganate	1.0 g/l	2.5, 5.0 and 10.0 min.
*Benlate (50%)	1.0g/l, 0.5 g/l	15.0, 24.0 and 48.0 h
*Captan (75%)	1.0g/l, 0.5 g/l	15.0,24.0 and 48.0 h
Sodium hypochlorite	50.0ml/l, 100.0ml/l	10.0 and 20.0 min.
*Activated charcoal	25.0g/l, 50.0 g/l	24.0, 1.0 and 0.5 h
** Bacitracin	1.0g/l, 0.25 g/l	5.0 min and included in the culture media.
*** Rifampin	0.1g/l, 0.050g/l	Included in culture media.
*** Mycostatin	0.1g/l, 0.050g/l	Included in culture media.

* Autoclaved

** Dissolved in water and ultrafiltered

*** Dissolved in a little alcohol and hot water and ultrafiltered.

Once the decontamination was finished, the roots were cut into 2 or 5cm lengths and incubated in flasks containing basal nutrient medium (see below).

VIABILITY TEST

Root tissue viability was assessed by reaction with tetrazolium bromide stain [3,-(4,5-Dimethylthiazolyl-2)-2,5-dipenyltetrazolium Bromide] (MTT), (see Towill & Mazur 1975) in 0.1M phosphate buffer at pH 6 (Dawson *et al* 1969). Roots belonging to different samples were cut transversally or longitudinally and placed in sterile Petri dishes (5 cm diameter), containing 2 ml of aqueous MTT solution (1.0g/l in DDW) and 5ml of 0.1 M phosphate buffer pH 6.0.

This gave a final concentration of MTT of 0.3g/l. Samples were incubated at 40°C. (darkness) for 2 h to develop the stain. Active/viable tissues stain pink to purple with this technique.

BASAL NUTRIENT MEDIUM

Nutrient media were used in liquid or solid form. The basal nutrient medium was composed of Murashige & Skoog 1962 (MS) salts. The sucrose concentrations used were: 0,0 g/l, 30.0 g/l,40.0g/l, and 60.0 g/l, while the NAA concentrations were 2.7×10^{-8} M, 2.7×10^{-7} and 1.1×10^{-5}.

The medium was always supplemented with $NaH_2 PO_4 . 2H_2O$ at 0.170 g/l and adenine sulfate at 0.040 g/l. The pH of the medium was adjusted to 5.8 with 0.1 N Na OH or HCI. Media were autoclaved for twenty minutes at 1.0 Kg/cm^2. Cultures were incubated in darkness at 29°C, 18°C or 25°C.

An orbital platform shaker was employed to agitate cultures in liquid medium. All aseptic manipulations were carried out in a laminar airflow cabinet and standard sterile procedures were employed.

RESULTS AND DISCUSSION

The degree of contaminaton of cultured roots was assessed after 3 and 7 days of culture and subsequently at weekly intervals.

Contamination was largely of bacterial origin whether liquid or solid media were employed. In solid media, contamination appeared to arise on the area adjacent to the roots, suggesting that contaminating was external rather than internal. Contamination was more intense in those flasks inoculated with brown and yellow roots (primary and secondary). The difference between older (brown/yellow) and newer (white) roots may be due to differences in the production of root exudates, secretions, mucilages etc. (Rovira *et al* 1979) with root age.

Fungal contamination was not a problem, when fungicides were included in the procedure. Omission of fungicide treatment resulted in a high fungal contamination. Treatment with $HgCl_2$ appeared essential to obtaining uncontaminated roots, especially with brown or yellow roots. This suggests there may be internal as well as external contamination of root explants.

The presence, rapidity and depth of colonisation of root tissues by fungi has been described (Charest *et al* 1984). When firstly, the roots were exposed to $KMnO_4$ and $HgCl_2$, separately, but treated with fungicides later, bacterial contaminations was always higher in $KMnO_4$ treated explants and in some cases contamination also developed more rapidly.

The incorporation of activated charcoal was justified by its adsorbent properties. It was believed that activated charcoal might inhibit bacterial growth by adsorbing bacteria or substances necessary to/promotive of, bacterial growth. No definitive assay of the effectiveness of activated charcoal was performed so its continued incorporation in the procedure was on purely theoretical grounds. NaClO is commonly used in tissue culture procedures to disinfect explants. No particular benefit was detected with oil palm root explants so its continued inclusion was justified more by its capacity to remove the actived charcoal adhered to the roots than by any lethal properties. In this way, the following aseptic procedure was developed that could later be adopted as routine:

Cl_2 Hg 1.0g/l, 2.5 minutes; Benlate + Captan 0,5 g/l, 15.0 hr; activated charcoal 25 g/l 60 minutes and domestic bleach 100.0 ml/l, 10 minutes.

With this procedure it was possible to obtain white roots (primary and/or secondary) without contamination at around 50 per cent. Nevertheless, brown or yellow roots always had a very high percentage contamination, generally 100%!

The viability test made after disinfection showed that roots remained alive, especially the white roots. The degree of staining of the central cylinder, more specifically the pericycle region, was used as the measure of root viability . With brown or yellow primary roots and, to a lesser extent, the yellow secondary roots, this staining was weaker declining remarkably after one week. This apparent difference in viability may be related to root age and its effects on lignification, level of reserves or level of metabolic activity etc. and/or a greater porosity for Hg Cl_2.

In the oil palm are found roots of diverse age, diameter, degree of lignification, colour, etc. therefore, these differences might reflect different grades of resistance to a specific disinfection procedure.

On the other hand, so that the MTT test would furnish repeatable results it was performed under standardized conditions of temperature, darkness, incubation time, type of root, and target tissue to be assessed. In the present study, these conditions gave repeatable results, especially in the control roots, the pericycle and root tip region of which stained a sharp pink-violet.

In the secondary white roots, it was observed that after aseptic procedure the viability test showed, almost always, negative results, with regard to the root tip staining, whilst longitudinal or transverse sections indicated staining in the pericycle region. This is an important aspect and must be considered when root tips are used for culture in liquid media. The first innoculations were made in the repli-dish system, using explants of about 2cm length and solid media. But successive contamination led to the idea of introducing antibiotics into the aseptic procedure.

In the belief that contamination was largely a surface phenomenon, roots were treated by immersion in a 1.0 g/l aqueous Bacitracin solution for 5 mins. prior to culturing. Following 1 week incubation at 29^{0}C brown roots again developed a high rate of contamination whilst white roots developed less contamination. Again, even in uncontaminated brown roots, a loss of viability as indicated by staining reaction, was observed. In regard to the white roots the results were more variable. The brown roots were observed to possess more air-spaces within the cortex than the white roots and it is possible that the compounds used in the aseptic procedure could reach the central meristimatic tissues more easily than in the white roots, which had a more compact cortex.

The effect of various media constituents upon root viability was examined in the following manner. Liquid medium and a filter paper bridge system to support the explants (primary and secondary white roots, 2cm length), was used. To a liquid (MS) medium was added: sucrose (60.0 g/l), NAA at 2.7×10^{-8}M or 2.7×10^{-7}M, with or without Bacitracin 0,250 g/l to evaluate its effect upon contamination. The flasks were incubated at 29^{0}C. The results, twenty days later, did not show any marked difference between treatments with or without antibiotic, in both cases, the contamination rate was approximately 60%. There was also no difference in the percentage viability between the treatments with NAA and without NAA, moreover the primary white root showed more viability than the secondary white roots. Browning occurred at the cut ends of the root explants. In some cases this browning advanced through the central cylinder.

It was thought that the high temperature used, (29°C), together with the high sugar concentration, (60.0 g/l), could have favoured phenolic oxidation, therefore another assay was performed in liquid media, but using activated charcoal (1.0g/l) sucrose at 40.0 g/l, and NAA 1.1 x 10^{-5} M, Bacitracin at 0.250g/l and white root explants of 5 cm length. Cultures were incubated at 18°C. As a control some flasks were incubated at 29°C. Similarly, some flasks were incubated without activated charcoal at 18 or 29°C to evaluate any effect of activated charcoal.

After three days the cultures incubated at 29°C, showed, without exception, an incipient opaque colour; However, those cultures incubated at 18°C showed no obvous contamination after 7 days. In this experiment no browning arose either in the controls or the treatments, suggesting that care in not damaging the roots was more effective than inclusion of activated charcoal in the medium.

After further incubating about 40 per cent of flasks kept at 18°C developed an opaqueness. A sample of those roots not indicating contamination gave a positive reaction to the MTT test.

To test whether this opaque colouration was caused by bacteria, flasks from the 18°C treatments were transferred to 29°C. During the following three days, 80 per cent of the flasks developed obvious contamination raising doubts about the efficacy of the Bacitracin treatment and impeding a correct interpretation upon the action of NAA.

Based upon this last assay it was thought that a combination of 29° and high sugar concentration is not advisable to achieve a low level of root contamination.

For this reason the next assay was done in three phases, using liquid MS medium. In the first phase MS medium was used with: NAA 1 1 x 10^{-5}M, activated charcoal 1.0 g/l, no sugar, but Rifampin and Mycostatin 0.1 g/l included. Each flask was inoculated with 4 explants. These were white primary or secondary roots cut to 5cms length. Flasks were incubated at 18°C, after one week, the colour of the media was not modified, and the MTT test demonstrated a high viability in most flasks. Browning was not observed even in the control flasks without activated charcoal.

In the second phase, the root explants were maintained in the same conditions, but sucrose was added to the medium at 20.0 g/l. Under these conditions, after one week, 60 % of the flasks remained apparently uncontaminated.

The inclusion of sugar in the media had a stimulatory effect upon the growth of contamination, but, this was not strong, probably because of the low incubation temperature and the inclusion of antibiotics in the media. The combination of these factors may have acted as a brake to bacterial growth.

In the third phase, those root explants remaining uncontaminated after 15 days incubation in phase two were transferred to another medium which contained the same foregoing characteristics, but, with sucrose at 40.0 g/l, NAA at 2.7×10^{-7} M, no activated charcoal and incubated at a temperature of $25^{o}C$ one root was inoculated per flask resulting in a total of twenty flasks, which were separated into two groups: one with and one without the antibiotics, Rifampin and Mycostatin, at a new concentration of 0.05g/l. By this stage the white primary roots had a better appearance than the white secondary roots and so were preferentially used in this stage. After twenty one days incubation it was observed that 80% of the flasks without antibiotic were contaminated. The medium of these flasks was opaque. In the other group, only approximately 20% appeared contaminated. The MTT-test indicated that uninfected roots were still alive.

The object of this investigation was to find an effective method of decomtaminating roots whilst still retaining their viability. No attempt was made to investigate suitable callus induction media. However, the information obtained by these experiments suggests the following conclusions:

1. The brown or yellow roots appear to be more heavily contaminated than the white roots.

2. The bacterial contamination appears more difficult to overcome than fungal contamination.

3. Benlate and Captan contributed effectively to the control of fungus in white and brown roots.

4. In decreasing order of apparent viability following decontamination procedures were: white primary and secondary roots, yellow primary and secondary roots, brown primary and secondary roots.

5. The MTT-test appears to be a good procedure to detect root viability, since it gives repeatable results under standardized conditions.

6. The MTT stain appeared to show a major affinity for meristematic regions of the root tips and central cylinder - specifically the pericycle region of root explants.

7. The mixture Rifampin-Mycostatin, was more effective than Bacitracin in the control of bacterial growth.

8. Pre-treatment by incubtion in MS liquid medium without Sucrose at 18°C appeared to weaken bacterial growth.

9. By using root explants of 5cm in length, browning was reduced independently of the presence of activated charcoal.

II. Callus induction and embryo germination.

Oil palm kernels from mature fruit brought from CNPSD-Manaus (Centro Nacional de Pesquisa de Seringueira e Dendê), were firstly broken, and the seed washed with tap water and soap, before being placed to imbibe in sterilized glass distilled water for 24h. Later, all seeds were surface-sterilised by soaking in 20% commercial bleach containing 0.05% (v/v) tween 20 emulsifier for 20 minutes. Embryos were removed aseptically with the aid of scalpels and forceps, inside a laminar flow cabinet and inoculated into flasks with 10ml of basal nutrient medium (MS), supplemented vith the following substances (g/l).

$Na\ H_2\ PO_4 .\ 2H_2\ O$ (0.170); adenine sulfate (0.040); sucrose (40.0); cassein hydrolysate (0.3); agar (6.0); activated charcoal (1.0) and 2,4 D 4.5×10^{-4}M. The pH of the medium had been adjusted to 5.8 with 0.1 N Na OH or HCl prior to the addition of agar and charcoal and the medium autoclaved at 1.0 Kg/cm for 20 minutes.

The inoculated flasks were incubated in darkness at 29°C

SUBCULTURE

The germinating embryos were transferred to conditions of 12 h light at 29°C. After seven weeks the shoots were transferred to other media to encourage growth and rooting. The basic nutrient medium was MS again, but with the addition of (g/l): Sucrose (30), NAH_2PO_4 . $2H_2$ 0 (0.170) and agar (6.0) the conditions of light and temperature were not modified. Embryos developing callus were transferred after seven weeks to basal medium supplemented with 2.5 x 10^{-6} M or 2.5 x 10^{-4} M 2,4 D and incubated at 29°C and 12 h light.

Results and Discussion

About 50% of embryos inoculated germinated after two weeks. Bad conditions of storage of the kernel and other causes linked to genetic factors may explain this low viability.

Of the remaining 50%, some were infected, some showed some tissue expansion but no root or shoot production whilst others showed no activity at all. Of those embryos that germinated 45% (22.5% overall) showed normal germination of shoot and root. 35% (17.5% overall) showed only shoot development whilst in 20% (10% overall) only the root emerged, this included embryos with shoots less than 2cm in length.

After 2 months root length varied between 1 to 5 cm, whilst shoot length varied between 2 to 5 cm. There results are similar to observations made in CNPSD, at least, as far as three forms showed by the embryos germinating under in vitro conditions.

It is possible that genetic factors, mechanical damage, or influences from the media are involved in this embryo behaviour.

In regard to callus formation after only fifteen days from inoculation incipient callus formation was already occurring. A lesser volume of callus formed on embryos on media with 2.5 10^{-4} M 2,4 D than on embryos incubated on 2.4 x 10^{-6}M 2.4 D. These latter calluses had a looser aspect than those developed on higher auxin which were more compact.

Fifteen weeks after inoculation some callus on 2.4 10^{-6} M 2.4 D obtained a diameter of approximately 1.0 cm, while others were smaller but with chlorophyll.

Unhappily some flasks of treatment 2.5 10^{-4} M 2.4 D were infected with bacteria some time after the first transfer. This problem was probably attributable to intake of unsterile air as the flasks were taken hot from the autoclave to be shaken in the laminar flow cabinet to mix the activated charcoal during cooling.

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