SHOOT REGENERATION FROM CALLUS DERIVED FROM EMBRYO AXIS CULTURES OF *Theobroma cacao in vitro*[1]

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Resumen

Se indujo la producción de callo de secciones de la plúmula de semillas maduras de cacao cuando se cultivó embriones axiales en el medio básico compuesto de Murashige y Skoog, suplementado con nitrato de calcio, sucrosa, peptona bactereológica, ácido neftalín acético, quitina e inositol. Subcultivos del callo después de 50, 60, 70, 80 y 90 días en el medio básico modificado con vitaminas blancas, diferenciaron gemas adventicias in vitro.

Introduction

In Nigeria, the cacao industry is currently faced with a prominent horticultural problem. This is the lack of an adequate rapid vegetative propagation method for cacao which is capable of making suppliers meet planting material demand pressures. This can be attributed to the rapidly expanding cacao growing areas and the giant rehabilitation scheme designed for improving old moribund farms. Accordingly, the tissue culture method is being explored with this problem in view. In recent years the application of tissue culture techniques has been extended to several plant breeding programmes. The ultimate aim has been to produce and rapidly propagate superior, uniform genotypes within a short time. To attain this, the induction and growth of organised structures have been attempted in somatic callus cultures of several tree species (5, 10, 13). Commercial feasibilities have been attained in the rapid clonal propagation of several herbaceous ornamental plants through the application of plant tissue culture technique (13). However, in many tree species, callus subculture and recultures have formed either shoots or roots and only occasionally both organs are differentiated in succession (2, 14). Nevertheless, comparatively fewer tropical fruit trees have been successfully induced, reared and maintained under tissue culture conditions. In this paper, a report is made of an attempt at vegetatively propagating cacao through *in vitro* method.

Materials and methods

Mature unripe fruits (pods) of *Theobroma cacao* L. were randomly collected from trees located at the Gambari Experimental Station of the Cocoa Research Institute of Nigeria, Ibadan. The pods were first surface sterilised by washing in lukewarm water at 35°C to which had been added some drops of 'Dettol' and antiseptic liquid and a few drops of 'Teepol' liquid detergent. These pods were further immersed in 0.5% sodium hypoclorite solution (10% Clorox) for 15 minutes; without further rinsing, each of the pods were cut open. The pod husk was separated from the mucilage placenta and seeds. The embryo axis of each mature bean (seed) were excised carefully and aseptically, using mounted blades on metal scalpels. Each embryo axis (explant) was quickly transfered on to a sterilised 10 ml modified Murashige and Skoog (10) basal medium

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contained in 30 ml McCartney specimen bottles. The modifying addenda of the medium used for callus induction (callogenesis) and regeneration are presented on Table 1. The cultures were stored under 14 hour diffuse daylight condition provided by Phillips fluorescent lamps of 150 – 250 lux at a temperature of 26 ± 2°C. The optimum auxin concentration (naphthalene acetic acid, NAA) for callus induction was determined in a preliminary trial (7). Eight concentrations examined were 0.0, 0.1, 1.0, 2.0, 3.0, 5.0, 10.5, 15.0 mg/l. A level of 2.0 mg/l concentration was chosen as the most satisfactory for callus induction from the plumule. Using this preliminary result as a guide, calli thus induced in this experiment were subcultured on the 50th, 60th, 70th, 80th and 90th days respectively. These subcultured calli were examined for possible incipient or latent organogenesis and/or embryogenesis microscopically. Callus originating from the plumule, the hypocotyl as well as the radicle ends were subcultured separately so as to compare and contrast their morphogenetic properties

Result

Callus was visible after 30 days in only those cultures that contained 2.0 or more mg/l of NAA. For concentrations of auxins lower than 2.0 mg/l of NAA, the embryo axis explant mostly germinated with or without production of callus. Callus induction occurred from three identifiable regions of the explants namely the root apex or radicle, the plumule and the hypocotyl regions. Callus induction was often earliest in the radicle region relative to the plumule and hypocotyl regions. Most callus was induced when NAA concentrations were above 5.0 mg/l. Accordingly, these showed rapid necrosis (browning and progressive death) earliest. Callus produced from tissues cultured on media that contained concentrations between 2.0 and 5.0 mg/l were most friable and showed occasional nodulation. This was particularly most characteristic of callus induced from the plumule region.

All ages of subcultures made at 50, 60, 70, 80 and 90 days respectively showed a high degree of variability both within and between age treatments, especially with respect to the number of adventitious buds and shoots, the time required for their development, and the number of leaves eventually produced (Figures 1, 2, 3 and 4).

Adventitious roots were not observed in any of the cultures. Shoots were produced in all ages of subcultures (plumule callus) except those subcultured from the 90-day-old callus. The observation made is represented on Figures 2 and 3.

Organogenesis was not observed in all ages of callus induced from both the radicle as well as the hypocotyl regions of the explant. Differentiated adventitious buds and shoots were not always similar to those characteristic of cacao grown in nature. They often appeared vestigial. The number of leaves and leaf-like structures produced per shoot varied with treatments and ranged from 1 to 5. The highest number of these leaves and leaf-like structures occurred in subcultures made from the 70-day-old callus (Figure 4). No roots were produced in any of the regenerated adventitious shoots (Figures 5, 6 and 7).

Table 1. Media used for callus formation and shoot regeneration. M-S salts modified to contain calcium nitrate* and organic fraction made to contain bacteriological peptone.

Addenda to Murashinge-Skoog (13) salts formulations	Callogenesis (ppm)	Shoot regeneration (ppm)
$Ca(NO_3)_2$*	300.00	300.00
Sucrose	50.00	20.00
Bacteriological Peptone*	100.00	10.00
Naphthalene acetic acid	2.0	0.01
Kinetin	0.01	1.0
Bacto Agar	8 000.00	8 000.00
Inositol	100.00	400.00
Nicotinic acid	—	1.00
Glycine	—	2.00
Pyridoxine HCL	—	1.00
Thiamine HCL	—	4.00
pH Adjusted with 1N NaOH	6.1	6.1

* $Ca(NO_3)_2$ and bacteriological peptone were supplemented to M-S formulation.

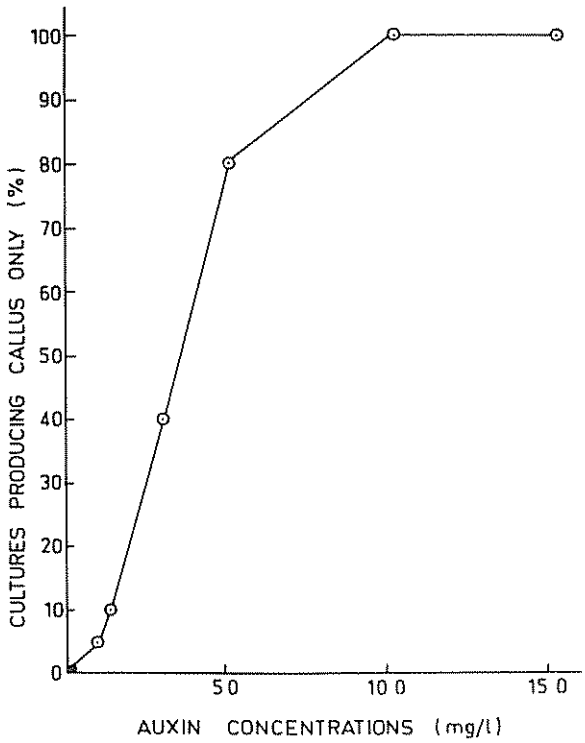


Fig 1. Effect of naphthalene acetic acid concentrations on callus induction in embryo axis explants.

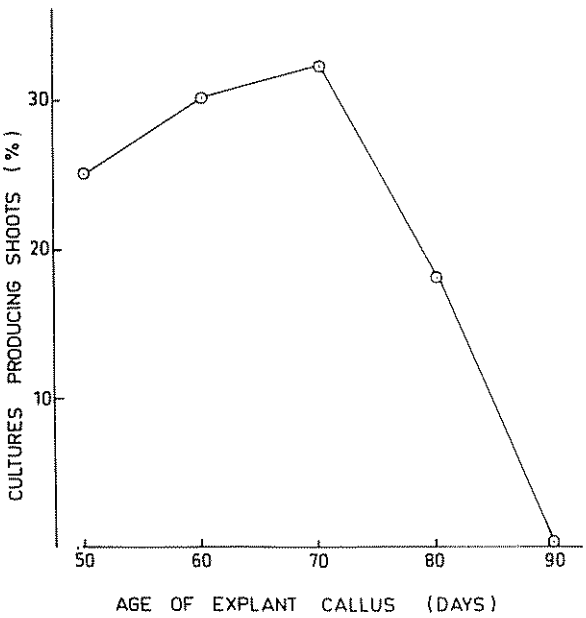


Fig. 2. Effect of age of callus induced from explant on shoot bud regnerability

Discussion

In this investigation, callus has been induced from one, two or three locations on the embryo axis explant depending on the auxin concentrations employed and the genotype of the seed. Adventitious buds and shoots of cacao were induced in callus initiated from mature cacao embryo axis explants.

Adventitious shoot regeneration from callus cultures has not been reported previously for *Theobroma cacao* L. or any other member of the family

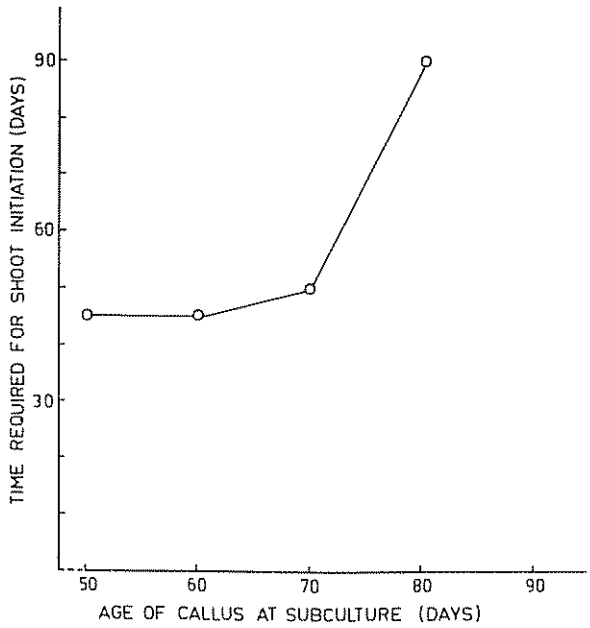


Fig. 3. Effect of age of callus induced from explant on the period of adventitious shoot initiation.

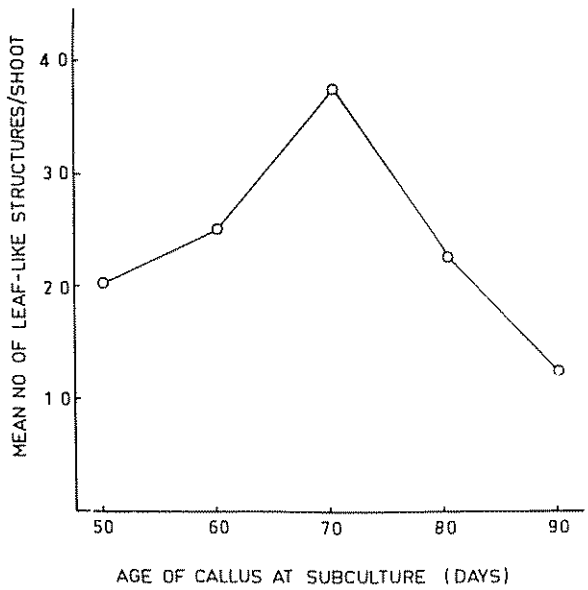
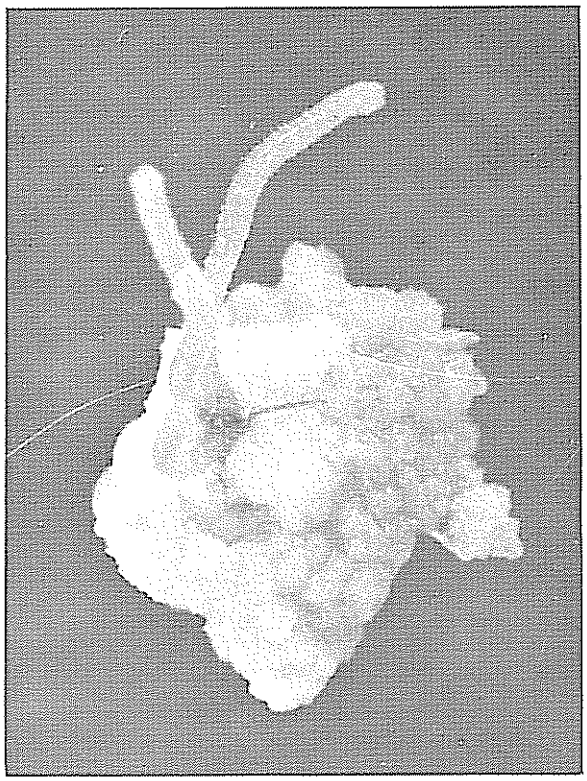
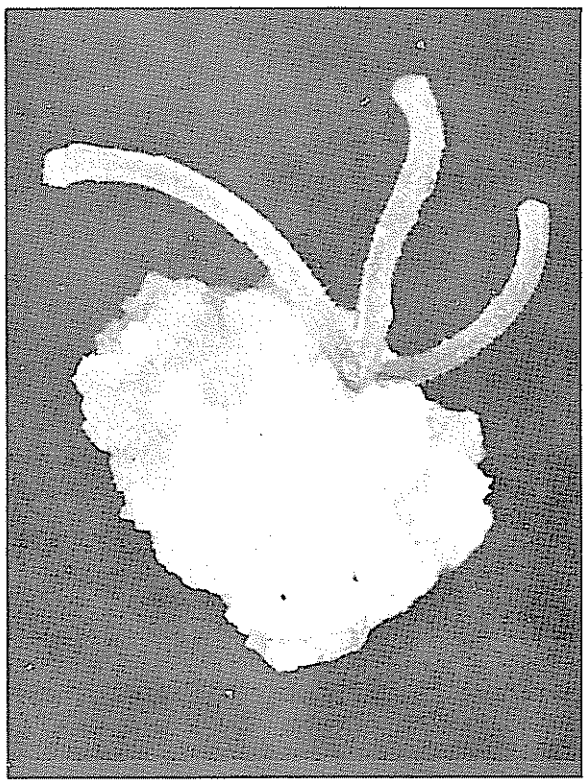
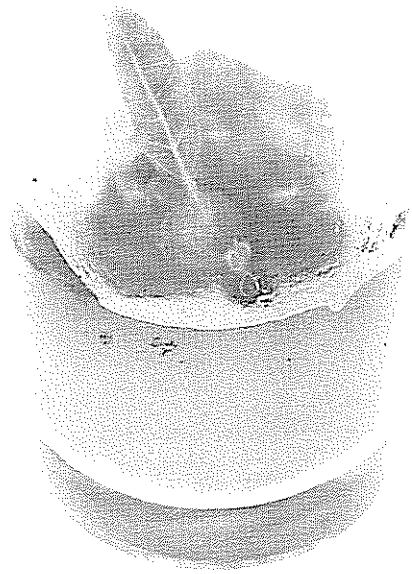


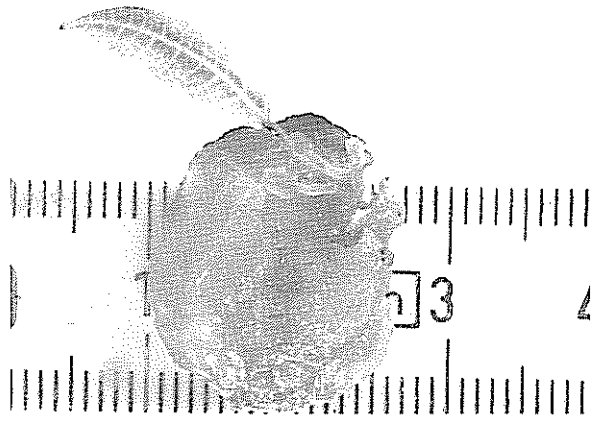
Fig 4. Effect of callus age on the number of adventitious leaves produced per shoot.





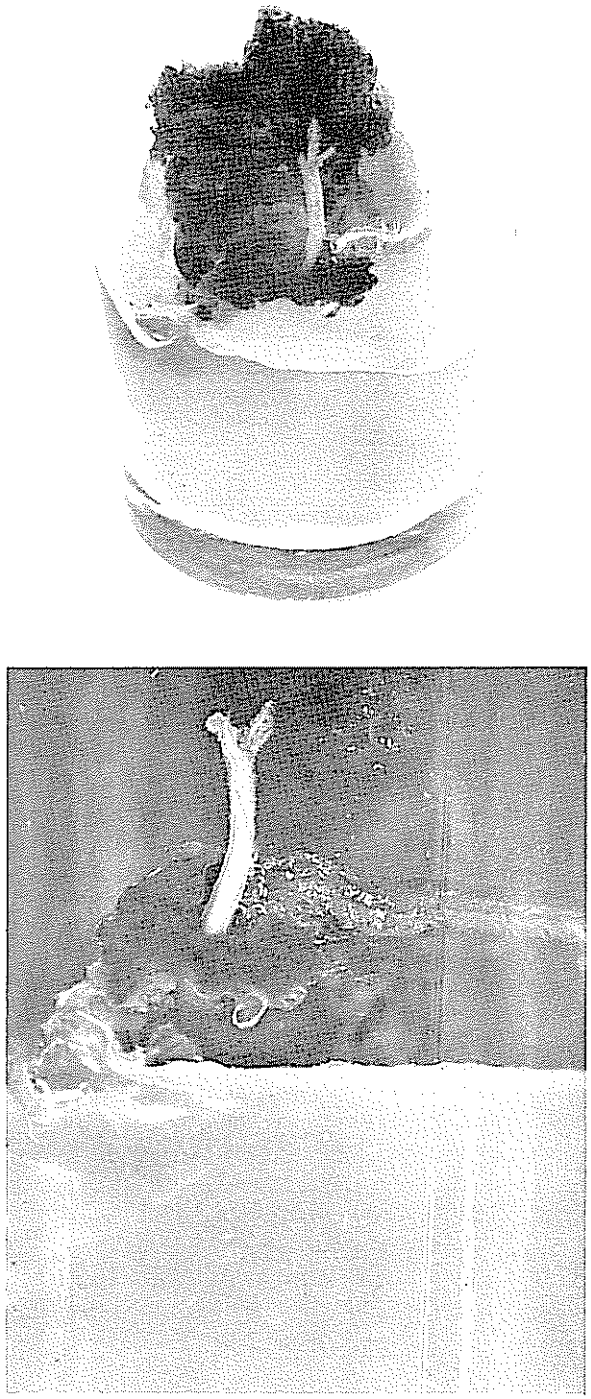
Figs. 5(a and b). Vestigial shoots from *Theobroma cacao* friable highly nodulated callus.





Figs. 6(a and b). A culture vessel containing callus from which a shoot has been produced. In (b) the morphogenetic callus and shoot are removed from their containers so as to show details.

sterculiaceae. However a few reports do exist on successful propagation of a few other non-forest, commercial fruit trees through the application of tissue culture methods. The first regularly sub-cultured callus cultures were reported for *Salix caperea* (8) and *Gastanea vesca* (9), while several other tropical fruit tree tissues of economic importance have been used to demonstrate inherent capability for organogenesis and/or embryogenesis among tree species. These species include *Citrus* and



Figs. 7(a and b) Culture bottles containing morphogenetic calli from which etiolated shoots are emerging. In both cultures, the callus has necrosed at the surface (turned brown) However adventitious budding is occurring from the fresh whitish callus at the base nearest the medium.
Note also a ball of callus being developed from the tip of the shoot in (b)

Citrus relatives (6), *Carica papaya* L. (4), *Coffea* species (16, 18). Nevertheless commerical feasibilities of these successes have not yet been attained. Furthermore, other fruit tree tissue cultures such as apples (11), avocado (1, 15), cacao (7)have even failed to respond organogenetically beyond rooting. Callus induction has been found to be easiest with embryos or sections from very young seedlings of tree species, and the most morphogenic callus have been produced from seed or seedling hypocotyl explants (13). This justifies the choice of the explant used in this investigation.

Observations in this investigation suggest that tissues in different parts, even when as small as the cacao bean embryo axis, tend to have different minimum auxin threshold for producing callus. This seems obvious in that while callus was readily induced in the radicle region, good shoot growth was still being enhanced at the plumule region even though both regions were in direct contact with the medium. A comparable observation, which was partly explained by polarity, has been described by Esan (6), when excised whole citrus nucellus was used as explants. The micropylar half remained embryogenic and produced nucellar embryos while on the other hand the chalazal region grew in a disorganised fashion and produced callus which also remained undifferentiated even after many subcultures and recultures.

Mehra and Mehra (12) had pointed out that even though equally fast growing calli are often obtained from different parts of the tree, the morphogenetic potentials of those calli often vary with the actual site of origin on the explant.

It is generally known that rhizogenesis *in vitro* occurs more readily than other forms of regenerative growths. Nevertheless shoot formation followed by rhizogenesis (rooting) has been reported to be characteristic of cultures that are freshly isolated from hypocotyls, stem meristems and young leaf segments (14). Accordingly, observations made in this investigation confirm this general claim.

This report also suggests that the age of callus being subcultured has direct effects on inherent morphogenetic potentials. Similarly, several workers have observed similar phenomena in callus cultures that have either aged or that had gone through several passages (17).

The adventitious shoots, which were differentiated in this investigation, have also been maintained for as long as 9 months without root development. Similar rootless shoots have been produced in callus cultures of some other plants such as *Pergularia* (3, 14, 19).

In conclusion, even though shoots have been regenerated from cacao callus cultures its practical importance and application for attaining the goal being aimed at is still far from being satisfactory. Nevertheless, this achievement will serve as a foundation for future work.

Abstract

Callus was induced from the plumule regions of mature cacao seeds when the embryo axis was cultured on a basal medium composed of Murashige and Skoog Salts, calcium nitrate supplement, sucrose, bacteriological peptone, naphthalene acetic acid, kinetin and inositol. When this callus was subcultured after 50, 60, 70, 80 and 90 days respectively on the basal medium modified to contain white's vitamins, adventitious buds were differentiated *in vitro*.

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