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Oil Palm (*Elaeis guineensis* Jacq.) Clonal Fidelity: Endogenous Cytokinins and Indoleacetic Acid in Embryogenic Callus Cultures

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ABSTRACT

Endogenous levels of indole-3-acetic acid (IAA) and cytokinins were measured in the two types of callus of oil palm (*Elaeis guineensis* Jacq.) by an HPLC–ELISA methodology. The Nodular Compact Callus (NCC) is commonly used to establish stable embryoid strains ensuring clonal fidelity (≤ 5% abnormal plants) while the soft and friable callus, called Fast Growing Callus (FGC), leads to the malformation of inflorescences in regenerated plants (100% abnormal plants). The most striking result was that no significant amounts of cytokinins were detected in FGC whereas they accumulated in NCC. These trends were reproducible during a subculture time. By contrast, IAA levels were not significantly different between each type of callus of the four clones tested. Endogenous cytokinin levels can be considered as markers of the physiological status of the callus, whatever the clone. There appears to be a close relationship between the ability of FGC to regenerate plants with abnormal inflorescences and low levels of cytokinins. The different mechanisms leading to the formation of FGC are discussed.

Key words: Oil palm, cytokinins, IAA, somatic embryogenesis, clonal fidelity.

INTRODUCTION

In vitro vegetative propagation of oil palm (*Elaeis guineensis* Jacq.) has been developed for 10 years in Africa and South East Asia on a large scale using somatic embryogenesis (Dublin, Enjalric, Lardet, Carron, Trolinder, and Pannetier, 1991). Embryogenesis was achieved through two different types of callus (Hanower and Pannetier, 1982). The first one, characteristically compact, organized and nodular (Pannetier, Arthuis, and Liévoux, 1981) was called Nodular Compact Callus (NCC). It has been used in industrial processes to establish stable embryoid strains ensuring a good clonal fidelity (Durand-Gasselin, Le Guen, Konan, and Duval, 1990). Soft and friable calli have also been described (Hanower and Hanower, 1984; Rabechault and Martin, 1976). These cultures, called Fast Growing Callus (FGC), arose from primary calli and could be kept for long periods of time.

Recently, the hypothesis was put forward that FGC is involved in the malformation of oil palm inflorescences, described as mantled (Duval, Durand-Gasselin, Konan, and Pannetier, 1988). The exact cause of this disorder remains unclear (Corley, Lee, Law, and Wang, 1986; Soh, 1987). It was only expressed in the reproductive organs and sex differentiation with a feminization of male parts. From the literature there is considerable evidence that sex expression is controlled by levels of endogenous hormones (Chailakhyan, 1979; Dauphin-Guerin, Teller,

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; MS = Murashige and Skoog culture medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = benzylaminopurine; PAS = periodic acid–Schiff; Z = zeatin; iP = isopentenyladenine; [9R]Z = zeatin riboside; [9R]iP = isopentenyladenosine; ABA = abscisic acid; [9R-5'P]Z = 5'monophosphate of [9R]Z; [9G]Z = 9-glucopyranosyl-Z; (diH)Z = dihydrozeatin; (diH)[9R]Z = 9-β-D-ribofuranosyl-(diH)Z; (diH)[9G]Z = 9-glucopyranosyl-(diH)Z; (diH)[9R-5'P]Z = 5'monophosphate of (diH)[9R]Z; [7G]Z = 7-glucopyranosyl-Z.

and Durand, 1980; Durand and Durand, 1984), especially in oil palm (Corley, 1976). The tissue culture process itself appears to induce alterations as the cells proliferate in a relatively undifferentiated state (Evans and Sharp, 1986; Phillips, Kaeppler, and Peschke, 1990). Because of the importance of growth substances in morphogenetic processes (Hanower and Hanower, 1984; Minocha, 1987; Williams and Maheswaran, 1986), it is hypothesized that levels of endogenous hormones in the calli could be involved in the induction of the malformation.

The aim of this work was to compare the two types of callus by measuring their auxin and cytokinin levels. We used an ELISA on HPLC-purified extracts from two types of callus, subcultured on the same media, to quantify simultaneously the amounts of IAA and cytokinins in the same material. Here, we show that IAA levels do not depend on the morphology of the callus while cytokinin levels can be considered as a valuable marker to distinguish normal oil palm plants from those which produce abnormal inflorescences.

MATERIALS AND METHODS

Plant material

Callus cultures from leaf segments were obtained as previously described by Pannetier *et al.* (1981). After about 100 d of culture, the primary calli were collected separately from explants and subcultured on agar-based (8·0 g dm^{-3} Agar-Agar, Sigma, USA) MS medium (Murashige and Skoog, 1962) supplemented with 2,4-D (100 mg dm^{-3}), BAP (1·0 mg dm^{-3}) and activated charcoal (3·0 g dm^{-3}, Sigma). pH was adjusted to 5·0. All the media constituents, including the growth regulators, were added before autoclaving at 110 °C for 20 min. Cultures were grown under 12 h light at 40 μE m^{-2} s^{-1}. Maintenance of cultures involved routine subculture of calli at 6-week intervals: this type of culture is described as Nodular Compact Callus (NCC). Sometimes, these calli gave rise to friable Fast Growing Callus (FGC). The FGC was isolated and subcultured at 3-week intervals. Ten days after transfer, the content of five culture tubes from each type of callus was harvested, weighed, frozen into liquid nitrogen and freeze-dried for 48 h. It was then weighed again to obtain the DW, powdered and kept at −30 °C until extraction for hormone analysis. The assay was repeated twice. A fresh-weight growth curve was established by weighing the calli taken from five tubes of each type at weekly intervals.

Four clones (designated C1, C2, C3, C4) each with two types of callus were studied. They were chosen because of their different origins and the different behaviours of the original trees in the fields. The C1 callus clone was obtained directly from a normal palm; the C2 callus clone was obtained from a normal palm regenerated from NCC; the C3 callus clone was obtained from an abnormal palm regenerated from NCC; the C4 callus clone was obtained from an abnormal palm regenerated from FGC.

Quantitative determination of endogenous plant growth substances

The immunoenzymic method described in detail previously (Maldiney, Leroux, Sabbagh, Sotta, Sossountzov, and Miginiac, 1986; Pelèse, Megnegneau, Sotta, Sossountzov, Caboche, and Miginiac, 1989; Sotta, Pilate, Pelèse, Sabbagh, Bonnet, and Maldiney, 1987) allowed the quantitation of IAA and several cytokinins, in the same extract. Among the cytokinins which cross-reacted with the anti-[9R]Z antibodies (Table 1), some ([9G]Z, Z, (diH)Z) were eluted in fractions 13–14 (Fig. 1). Therefore, the immunoreactivity detected in these fractions will be referred to as cytokinins 13–14. In brief, freeze-dried powdered tissues were stirred up and extracted with cold 80% methanol containing 40 mg dm^{-3} butylhydroxytoluene as antioxidant for 60 h at 4 °C in darkness. Tritiated radiolabelled standards (IAA and ABA, specific activity 27 GBq $mmol^{-1}$ and 79 GBq $mmol^{-1}$, respectively; Amersham, UK) were added to the samples for recovery estimation after purification: 0·11 kBq for IAA, 0·6 kBq for ABA. IAA and ABA have been shown to perform as internal standards for cytokinins (Maldiney *et al.*, 1986; Sotta *et al.*, 1987); moreover radiolabelled cytokinins are not commercially available. The methanolic extracts were filtered and then passed through Sep-Pak C18 cartridges (Waters, Milford, USA) eluted with 80% aqueous methanol. The eluates were reduced to water *in vacuo* with a rotary evaporator and adjusted to pH 3·0 with diluted formic acid; then submitted to a HPLC fractionation through a reverse phase column (ODS, Lichrocart, Merck, FRG) fitted on a Beckman (USA) HPLC apparatus and eluted with a 53 mM formic acid/methanol gradient. Triethylamine (5·0 mM) was added to improve separation. The fractions were collected (0·8 cm^3, 1 min elution time) and reduced to dryness in a Speed-Vac concentrator (Savant, USA). They were then methylated with ethereal diazomethane for IAA analysis prior to being submitted to ELISA with anti-IAA antibodies. Cytokinin quantitation was carried out by ELISA with anti-[9R]Z and anti-[9R]iP antibodies. The level of each hormone in each sample was measured five times and the values were corrected for recovery. The results were subjected to an analysis of variance in order to determine the significance of the difference between the two types of callus for each clone (callus effect).

Histological techniques

The different types of callus of each clone were fixed for 24 h using 10% paraformaldehyde diluted in 0·2 M phosphate buffer (pH 7·2). Tissues were then rinsed in a 70% ethanol solution. After rinsing, dehydration and impregnation in methylmethacrylate, each sample was embedded in the polymethylmethacrylate LKB Historesin (Sweden). Polymerization was performed for 2 h at room temperature (Schwendiman, Pannetier, and Michaux-Ferrière, 1988). Semi-thin sections (2·0–3·0 μm thick) were obtained with an ultramicrotome (Historange, LKB) equipped with steel knives and spread on slides. Staining was performed with a PAS reaction supplemented with Naphthol blue-black.

TABLE 1. *Specificity of anti-[9R]Z antibodies*

The cross-reaction is calculated as the percentage ratio of the molar concentrations of [9R]Z and the tested compound, at 50% binding.

Cytokinins	Cross-reactivity (%)
[9R]Z	100
[9R-5'P]Z	98
Z	90
[9G]Z	52
(diH)Z	12
(diH)[9R-5'P]Z	2
(diH)[9R]Z	1
(diH)[9G]Z	0·5
[7G]Z	0

RESULTS

Comparative structure of calli

The two types of callus of the four clones were subcultured on the same media. Their morphology and physiological status were different, but they exhibited a similar capability for embryogenesis.

NCC were nodular and showed an organized structure (Fig. 2A). The main characteristic of this type of callus was the differentiation of a cambium-like zone which ensured its proliferation. There was no visible epidermis. The outer cells degenerated at the callus periphery, whereas, in the callus centre, vacuolated parenchyma cells and vessel elements were found (Fig. 2B). Phenolic compounds were usually present in the parenchyma centre.

FGC arose spontaneously at a very low frequency from limited sectors in established callus cultures. They were characterized by their soft, white and friable appearance (Fig. 2C) and showed many sparsely, vacuolated and enlarged degenerative cells. No phenolic compounds were found. Growth of the calli was ensured by a few scattered groups of small cytoplasmic-rich cells which constituted meristematic clumps (Fig. 2D). Although no definite determination of cell numbers could be made in this type of culture, it appeared to contain a higher number of enlarged and vacuolated cells than meristematic cells.

Dry weight : fresh weight ratio for FGC was 1 : 15 and 1 : 10 for NCC. A major characteristic of the FGC was that they grew faster than NCC. Moreover, NCC stopped their growth after 6 weeks on the same media by contrast with FGC. Harvest of the culture tubes was made 10 d after the transfer when an increase in fresh weight of all the types of culture (Fig. 3) was achieved.

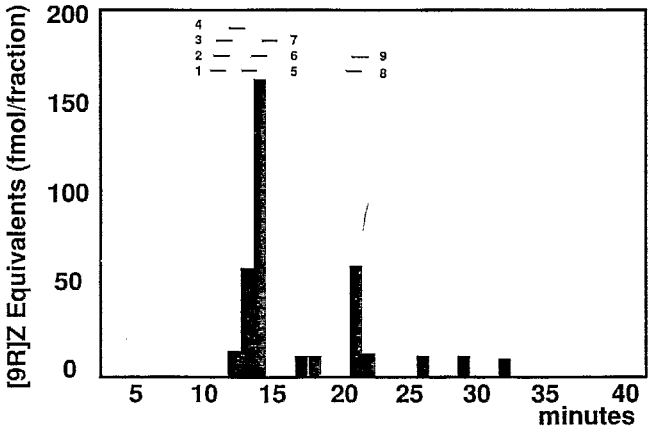

FIG. 1. Distribution of immunoreactivity detected with anti-[9R]Z antibodies in HPLC fractions of a purified extract (NCC, C3). Typical elution zones for several cytokinins (1 = [9R-5′P]Z; 2 = (diH)[9R-5′P]Z; 3 = [7G]Z; 4 = [9G]Z; 5 = (diH)[9G]Z; 6 = Z; 7 = (diH)Z; 8 = [9R]Z; 9 = (diH)[9R]Z) were determined with the UV absorbance at 270 nm and represented with horizontal bars. Each fraction corresponded to 0·8 cm^3 (1 min elution time).

Comparative hormone levels

IAA contents: There was no significant difference in IAA levels between the two types of callus, except within the clone C3 ($P < 0{\cdot}001$), derived from an abnormal individual (Fig. 4). FGC of C3 contained 6-fold more endogenous IAA than NCC. Within the three other clones, IAA quantities in the two types of callus were similar: there was no significant effect of callus type.

This result clearly indicates, except within C3, that differences previously described between the two types of callus cannot be correlated with endogenous IAA levels.

Z-type cytokinins: In FGC, both cytokinins 13–14 and [9R]Z levels were always close to the limit of detection (approximately 3·0 fmol per assay) of the immunoassay used. In NCC, both cytokinins 13–14 (Fig. 5) and, to a lesser extent [9R]Z, were measured in detectable levels (Fig. 6).

Within all the clones, the levels of cytokinins 13–14 measured in FGC were significantly different from NCC ($P < 0{\cdot}001$): cytokinins 13–14 concentrations in FGC were always lower than those measured in NCC, whatever the clone. There was a significant effect of callus type. Endogenous cytokinins 13–14 levels appear as markers of physiological status of the callus, whatever the clone.

Except within the clone C2, derived from a normal individual, there was a significant difference ($P < 0{\cdot}001$) in the levels of [9R]Z found in the two types of callus. [9R]Z content was higher in NCC than in FGC.

iP-type cytokinin: In the two types of callus, both iP and [9R]iP levels were very low and close to the limit of detection (approximately 10 fmol per assay). There was no significant difference between the two types of callus, whatever the clone.

iP-type cytokinin variations were not closely related to the type of callus by contrast with Z-type cytokinins. This result indicates that iP-type cytokinins could not be considered as markers to differentiate between the two types of callus.

Ratio IAA/cytokinins 13–14 + [9R]Z: The IAA to cytokinins 13–14 + [9R]Z ratio (Table 2) was significantly lower in NCC than in FGC independent of the clone. There was clearly a close relationship between callus type and hormonal balance.

Endogenous levels of Z-type cytokinins during subculture

We studied the variations of endogenous levels of cytokinins 13–14 + [9R]Z in the two calli of the clone C3 during the subculture (Fig. 7), to check the stability of the levels of these regulators. Endogenous levels of cytokinins 13–14 + [9R]Z did not change in calli that were taken 1, 4, 7, 10, 30 and 60 d after transfer on fresh medium. During this time, the white friable calli kept their appearance. Microscopic examination of the cells revealed that the number of large vacuolated cells

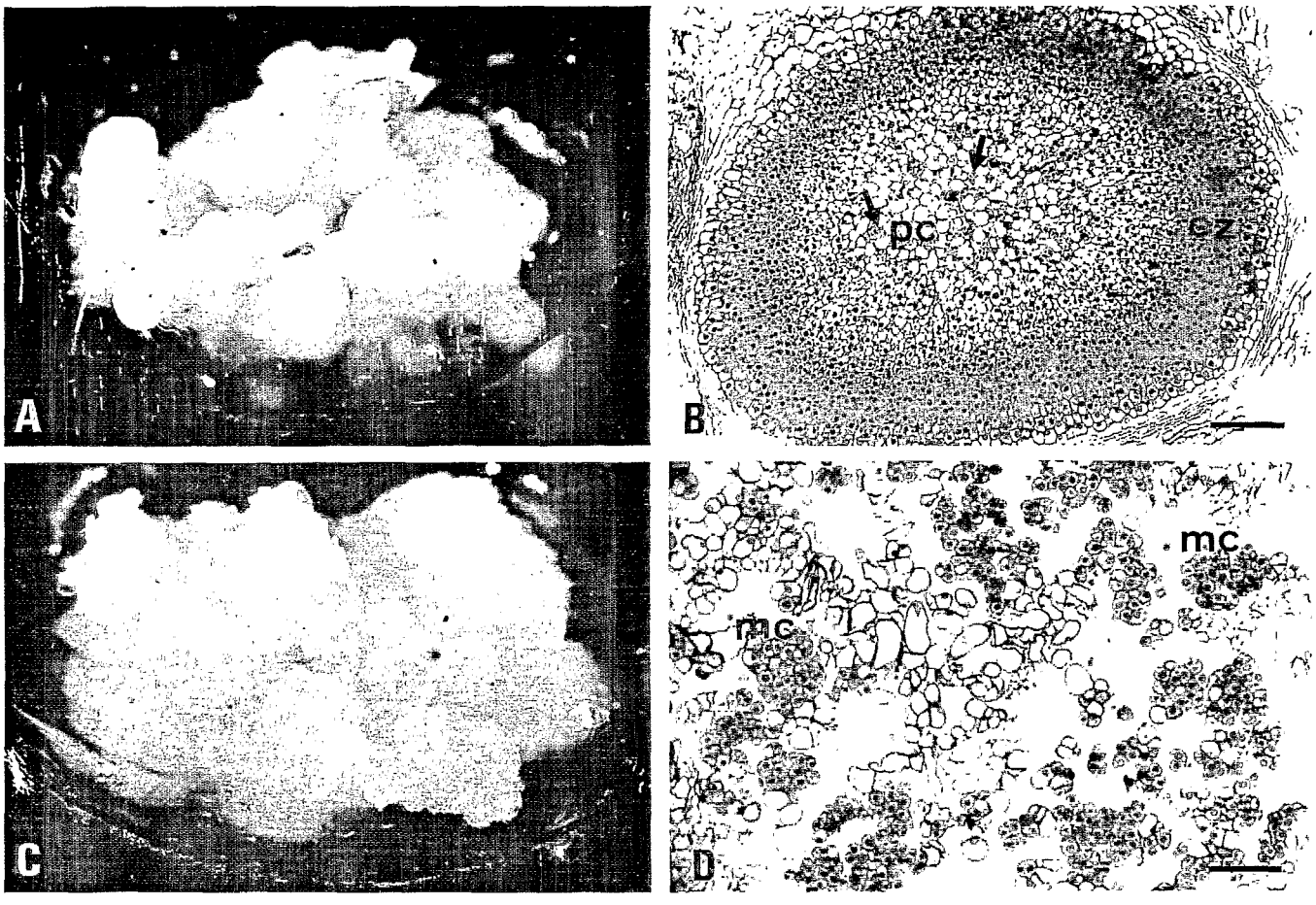

FIG. 2. Comparative structure of oil palm calli of clone C1. (A) Nodular Compact Callus (NCC) cultured on charcoal medium for 30 d. (B) Light micrograph of nodular callus tissue showing a cambium-like zone (cz) and regions with parenchyma cells (pc) containing phenolic compounds (arrows). (C) White friable calli (FGC), cultured on charcoal medium and obtained from limited sectors of nodular calli that were cultured on charcoal medium. (D) Light micrograph of friable callus tissue showing regions with meristematic cells (mc) and large zones of vacuolated cells more or less degenerative. Bar represents 50 μm.

increased with time, contributing to the large increase in fresh weight.

DISCUSSION

The two types of callus, already described in a great number of monocots (Vasil and Vasil, 1985; Wang, Huang, He, and Hao, 1990) differ completely on the basis of their morphology and their hormonal content. Data obtained in this study clearly indicate that the general levels of cytokinins 13–14 found in FGC are very low as compared to NCC, irrespective of the clone. In contrast, IAA levels are not significantly different between the type of callus from each clone. Therefore, tissue disorganization is closely associated with an abnormally high auxin–cytokinin ratio, as previously reported in Graminae (Vasil, 1987) whatever the origins of the clones. It is well known that the regulation of growth and differentiation involves complex interactions between growth substances (Wareing, 1977). Thus, we can hypothesize that the exogenous auxin-cytokinin equilibrium of the medium was the stimulus responsible for the random formation of FGC on NCC. This new morphogenetic feature could be induced by variations of constituents of the culture medium. The variable experimental conditions may induce variations in auxin concentrations (Ebert and Taylor, 1990) which are known to control both friability of calli (Evans, Sharp, and Flick, 1981; Murashige and Skoog, 1962) and endogenous cytokinin levels (Palni, Burch, and Horgan, 1988). Thus, a large variation of a component in the medium could be responsible for an alteration in gene expression resulting in a cellular disorganization associated with a high endogenous auxin–cytokinin ratio. It could explain the spontaneous and random formation of FGC on NCC. Therefore, according to the hypotheses of Meins (1989) concerning cell-heritable variations, the procedure of tissue culture itself could induce the formation of FGC through an epigenetic mechanism that generates heritable variations through successive mitotic cell generations.

Statistical analysis showed that only cytokinins 13–14 concentrations could be considered as markers of the

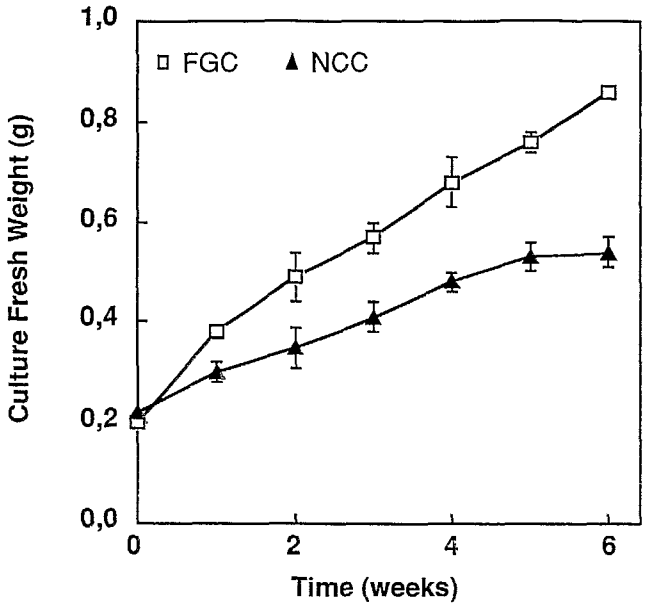

FIG. 3. Growth of oil palm calli of the clone C1. The number of cultures sampled was 5 for each harvest. Bars show ± s.e. of means.

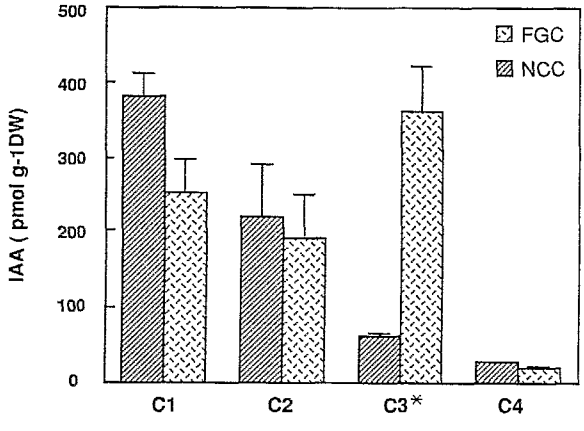

FIG. 4. Comparative endogenous IAA concentrations in NCC and FGC from four different clones. Data are means of 5 replicates ± s.e. Significative intra-clonal differences are indicated by $*P<0{\cdot}001$.

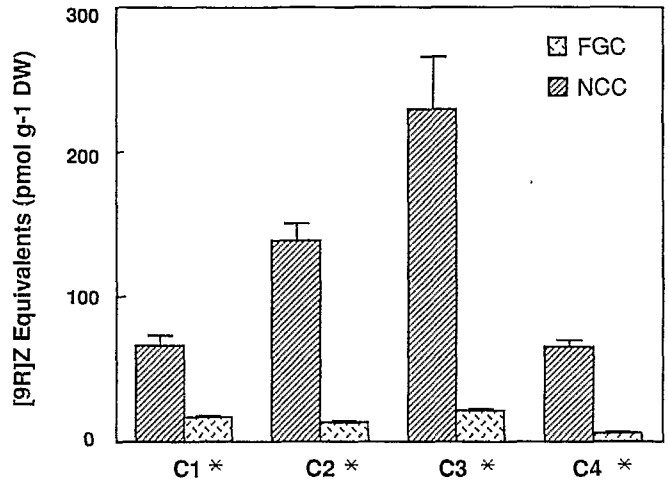

FIG. 5. Comparative endogenous cytokinins 13–14 concentrations in NCC and FGC from four different clones. Data are means of 5 replicates ± s.e. Significative intra-clonal differences are indicated by $*P<0{\cdot}001$.

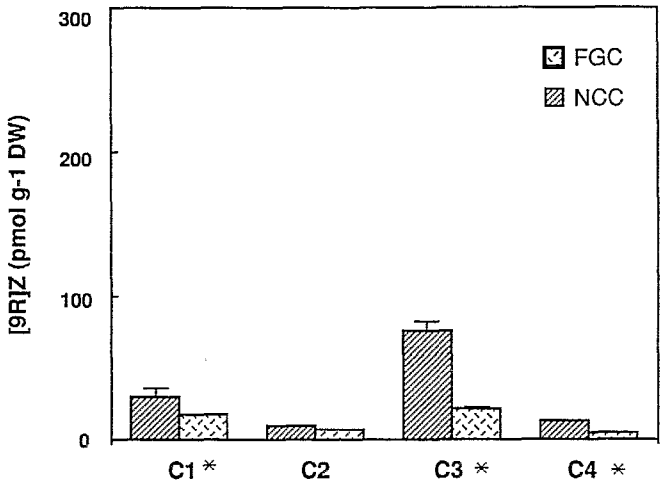

FIG. 6. Comparative endogenous [9R]Z concentrations in NCC and FGC from four different clones. Significative intra-clonal differences are indicated by $*P<0{\cdot}001$.

TABLE 2. *IAA to cytokinins* ratio of oil palm calli from four different clones*

*[9R]Z + cytokinins 13-14.

Clones	Callus type	
	NCC	FGC
C1	4·0	7·1
C2	1·5	9·7
C3	0·2	8·3
C4	0·4	1·8

difference between the two types of callus, whatever the clones. It is worth noting that among these, cytokinin Z is considered to be the biologically active form of the cytokinin (Laloue and Pethe, 1982). Cytokinins play an important role in cell division *in vitro* and are involved in stimulating cell division prior to embryogenesis (Letham and Palni, 1983). The striking result in this study was that cytokinins were present at very low levels in FGC which is characterized by fast growth. Different hypotheses could explain this cytokinin deficiency. One possible explanation is that cytokinins are effective in promoting growth at concentrations below the limit of detection of the assay. However, the difference between the two types of callus on the basis of their growth rate negates this hypothesis. Another possibility is that an entirely different class of compounds which do not cross-react with the antibodies used, is effective as a cell division factor. It is also possible that the cytokinin degradative mechanism is de-regulated.

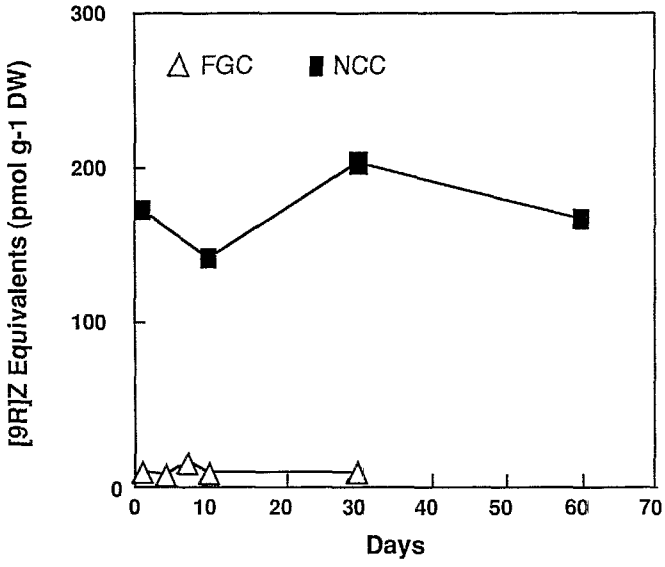

FIG. 7. Changes in cytokinins 13–14+[9R]Z levels during subculture of oil palm calli of clone C3.

Hormonal studies carried out on normal and abnormal immature female inflorescences, sampled from 3-year-old palms, have shown lower levels of cytokinins in abnormal individuals compared to normal ones (unpublished results). We can draw a parallel between these results and those obtained on calli, where FGC, considered as being responsible for the appearance of abnormality (Duval *et al.*, 1988), contained less cytokinin than NCC. Therefore, it is likely that the malformation of oil palm inflorescences is a physiological disorder associated with tissue disorganization and abnormally low endogenous cytokinin levels. Cellular and molecular conditions favourable to clonal fidelity in oil palm tissue culture are put forward.

It was reported that oil palm cultures do not require exogenous cytokinins to maintain cell division (Hanower and Hanower, 1984; Jones, 1990). It can be supposed that subcultured FGC were similar to cytokinin-habituated tobacco pith cells (Meins, 1989) with very low endogenous cytokinin levels. In this case, we have to know if FGC in our culture conditions require BAP to regenerate palms. Habituation can be due either to genetic change in culture or can be an epigenetic phenomenon (Meins, 1989). The perturbation is of a transient nature and some reversion of abnormal individuals to normal ones are observed in the field (Durand-Gasselin, personal communication). Therefore, it is unlikely that the formation of FGC is the result of genetic alterations. This hypothesis has to be confirmed by genetic evaluation requiring several sexual generations.

It is still not clear whether the somaclonal variation is the result of genetic heterogeneity pre-existing in the somatic cells of a plant or is induced by constituents of the culture medium. However, the system NCC/FGC provides a real model to study possible somaclonal variation.

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