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Cellular responses of leaf explants of *Cocos nucifera* L. *in vitro*

J.H.F. Jesty & D. Francis
School of Pure and Applied Biology, University of Wales College of Cardiff, P.O. Box 915, Cardiff CF1 3TL, UK

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Abstract

Leaf explants of *Cocos nucifera* L. (coconut palm) were studied *in vitro* in order to establish whether or not rapid cellular changes contribute to the well known recalcitrance of coconut cells in tissue culture. Segments from the base of immature leaves were cultured on modified Eeuwens' medium at 30°C in darkness. The mitotic index, nuclear DNA amounts, cell and nuclear size were measured both before and during culture (from 0 to 70 days). There was no basipetal gradient of cell division in immature coconut leaves; the mitotic index never exceeded 2% and showed neither a positional nor temporal relationship with leaf development. Moreover the vast majority of cells were in G1 of the cell cycle. This cell cycle pattern was maintained for most of the period in culture although at 70 days there was an increase in the proportion of cells in S- and G2-phases consistent with low rates of callus formation. The nuclear : cell size ratio was constant in cells within the immature leaf irrespective of developmental age. However upon transfer to culture media, cell size but not nuclear size increased. We suggest that this uncoupling of cell and nuclear size disrupts cell co-ordination and is a key contributor to recalcitrant cellular behaviour of this species *in vitro*.

Introduction

Cocos nucifera (coconut palm) is one of the world's top five oil-producing plants and is grown as a subsistence crop in all lowland tropical areas [1]. *C. nucifera* is a heterozygous outbreeder and consequently the nuts are genetically very variable. The selection of high yielding and disease resistant palms is therefore difficult. Thus, clonal propagation of *C. nucifera* through tissue culture is an attractive proposal but, to date, has met with limited success. Most reports of coconut tissue culture have concentrated on defining physical and chemical optima for sustained growth of explants *in vitro* [2, 3, 4]. For example, considerable cell expansion occurred in subapical stem explants from mature Malayan Dwarf palms when cultured on a modified Heller and Murashige & Skoog (MS) medium containing 10^{-7} M 2,4-dichlorophenoxyacetic acid (2,4-D) and 5×10^{-6} M kinetin. However, whilst proembryo-like structures arose their exact origin was not confirmed by a steriological study [5]. Moreover although media compositions capable of supporting the growth of zygotic embryos are available, a reliable method for obtaining somatic embryogenesis has yet to emerge for *C. nucifera*.

Our inability to manipulate *C. nucifera* cells *in vitro* relates to insufficient knowledge of their cellular behaviour and evaluating the application of plant growth regulators to cultures has been largely empirical. This, in turn, suggests that more primary information on the developmental state of the cells is required both prior to and immediately following culture. One approach has been to study the cell cycle status of cells *in vivo* and *in vitro* [6–8]. Cells of many grasses and

cereals become unresponsive *in vitro* at a very early stage in their development [6]. A gradient of cellular competence exists which corresponds to the developmental, and age, gradient along the length of the gramineous leaf. For example, in monocots such as *Lolium multiflorum* and *Triticum timopheevii*, growth is restricted both temporally and spatially to the mitotically active basal regions of the mature leaf [6, 9]. Given the recalcitrance of *C. nucifera* cells *in vitro*, it is important to establish whether a basipetal gradient of cellular differentiation exists in leaves which are normally used for *in vitro* studies. Moreover, using the *in vivo* data as a framework, cellular behaviour following transfer of leaf explants to culture media can be analysed.

Thus, the aims of the work reported in this paper were three-fold. First, we established whether or not a basipetal developmental gradient existed along the base of the coconut leaf. Second, the mitotic index and nuclear DNA content were measured in immature leaves both before and after the start of culture. Third, the relationship between nuclear and cell size was determined both *in vivo* and early, during *in vitro* growth.

Materials and methods

Plant material and greenhouse management

Unless otherwise stated, the plants were grown at 30 ± 5°C and 80% relative humidity. The coconuts (*Cocos nucifera* L. 2n = 2x = 32) used in this study were derived from a commercial plantation in the Solomon Islands (Lever Solomons Ltd) and were Malayan Dwarf × Rennell hybrids. Each nut was germinated by slicing across the largest of its three segments and soaking it in water for 24 h. The nuts were placed in a sand-seed bed so that 1/3 was above the sand, with the sliced surface uppermost. Each day, the palms were watered and when they were 6 months old, they were fed with liquid fertilizer. Either 6-, 12- or 14-month-old palms were used in this study.

In vivo analyses

The stem (ca 40 cm above the bole) was sawn off and discarded. The nut and remaining stem was removed from its pot. The nut was sawn off and discarded and successive leaves were removed acropetally; as each leaf was removed the root tissue was discarded. The stem was dissected as above until the spear leaf was exposed. From then onward, the spear leaf was defined as leaf 0, and successively younger leaves as leaf-1, leaf-2 etc.

For the *in vivo* work six palms were used: the basal 3 cm of leaf-1 of six-month-old palms: A, B, and C, was dissected into 3 × 1 cm transverse segments. Similarly, leaf-2 of 14-month-old palms: 1, 2 and 3, was dissected into 5 × 1 mm transverse segments. Note that whilst leaf-1 was, on average 40–50 cm-long, leaf leaf-2 measured about 15 mm. All dissected material was fixed in 3:1 absolute ethanol:glacial acetic acid and stored in a refrigerator at 5°C (≥24 h).

This study confined itself to basal leaf segments of immature leaves because basal leaf tissue is more likely to initiate callus growth [10] and it is a source of a large amount of concertina-like tissue, or plications [11]. Moreover, leaves-1 and -2 were the routinely sampled leaves for coconut tissue culture work at Unilever Research, Bedford, UK and, hence, were the start-point for our work.

In vitro analyses

Unless otherwise stated, 60 cm^3 Sterilin pots were used to maintain the leaf explants. A modified Eeuwens' [2, 3] medium, Y3, was used in this study. It comprised the *in vivo* and *in vitro* elements of the Eeuwens' recipe supplemented with: sucrose (2×10^5 μM); FeEDTA (100 μM) meso-inositol (566 μM); thiamine HCl (3 μM) pyridoxine HCl (5 μM), nicotinic acid (8 μM) glutamine (685 μM); asparagine (667 μM); arginine and 2,4-D (both 2.3×10^{-7} μM) gelled by 4 g l^{-1} agar (Oxoid #3).

Leaf tissue from three 12 month-old palms, DR2, DR21 and DR25, was dissected aseptically and cut as above and placed in a sterile solution of ascorbic and citric acid (both 100 mM) to prevent the tissue browning. The cut segments

were divided up into the individual plications and each was firmly placed onto medium. Cultures were maintained in the dark at 30 ± 1°C and transferred to fresh medium once every 6 weeks.

Cytological measurements

All fixed material was stained by the Feulgen reaction and permanent squashes were prepared [12]. The mitotic index (percentage frequency of cells in prophase, metaphase, anaphase or telophase) was determined by counting at least 1,000 cells in a series of random transects across the slide.

For the *in vivo* analyses, a minimum of three slides per segment were scored, for the first three leaf segments from palms A, B and C, and the first five segments from palms 1, 2 and 3.

For the *in vitro* work, the mitotic index was determined on squash preparations for days 0, 1, 2, 3 and 7 on leaf explants derived from the 12-month-old palms: DR2, DR21 and DR25. After a few days *in vitro*, the leaf tissue expanded and hardened which made squash preparations difficult. Therefore, mitotic index measurements were subsequently made on days 14, 28 and 70 on sectioned material using standard procedures [12].

Microdensitometry

DNA contents of Feulgen-stained nuclei were measured using a Vickers M85A scanning microdensitometer at 560 nm on days 0, 14, 28 and 70 on sectioned material from palms DR2, DR21 and DR25. On each slide, the absorbance of 10 half-telophase and 10 prophase nuclei were taken as internal standards for the 2C and 4C DNA values, respectively (where 1C is the nuclear DNA amount in the unreplicated haploid genome). Fifty interphase nuclei were measured in random transects. The DNA histograms were quantified using the methods of Evans & Van't Hof [13] in which nuclei with 1.6–2.4C, 2.4–3.6C, 3.6–4.8C, and >4.8C nuclear DNA amounts were taken to represent G1, S, G2 and polyploid nuclei, respectively. The provision in this analysis is that microdensitometric measurements of nuclei in S-phase tend to be underestimates which are normally quantified by labelling with methyl – ^{3}H – thymidine; coconut cells are difficult to label with this radioactive nucleoside [S.W. Armstrong & D. Francis, unpublished data; 14].

Cell and nuclear areas

Samples taken as described above were stained by the Feulgen reaction and counter-stained with light green (0.2% w/v in absolute ethanol). For the measurements of cell and nuclear area from the *in vivo* material cell monolayers were prepared so as not to squash the cells [12]. For the cells measured from the *in vitro* material, sections were cut as described above. Mitotic cell area, interphase cell and nuclear area and prophase nuclear area were measured on a Graphpad graphics tablet linked to a video unit. The results were calibrated in μm^2 using a BBC microcomputer interfaced to the graphics tablet.

Results and discussion

Mitotic index

The mitotic index data for leaf-1 and -2 are given in Table 1. All values lie within the range 0.53 ± 0.03 to 2.2 ± 0.2% and there was no gradient of mitotic indices along the leaf. Leaf segment position had no significant effect on the mitotic index although there was variation between individual

Table 1. Mean mitotic index (±standard error of the mean) for leaf segments dissected basipetally from leaf-1 from A, B and C ($n = 5$) and leaf-2 from palms 1, 2 and 3 ($n = 3$).

Leaf segment (Leaf-1)	Palm A	B	C
1	2.20 ± 0.20	1.8 ± 0.10	1.40 ± 0.20
2	2.20 ± 0.20	1.40 ± 0.70	1.30 ± 0.05
3	2.10 ± 0.40	1.30 ± 0.40	1.50 ± 0.20
(Leaf-2)	1	2	3
1	1.99 ± 0.47	1.03 ± 0.24	0.53 ± 0.03
2	-	1.10 ± 0.10	1.08 ± 0.10
3	1.41 ± 0.22	0.85 ± 0.31	0.79 ± 0.26
4	0.90 ± 0.11	0.86 ± 0.17	0.80 ± 0.05
5	1.08 ± 0.38	0.90 ± 0.26	0.76 ± 0.29

palms at the 1% level (see Table 2 for an analysis of variance for these characters). This is because the mitotic indices for palm A were higher than those for B and C (Table 1). However, the pattern of mitotic index was the same in each and, hence, there was no significant interaction between leaf segment and palm (Table 2). Overall, there was no basipetal gradient of cell division along the leaf. Because of this, subsequent samples for *in vitro* studies were simply taken from basal leaf tissue.

Mean mitotic indices for cultured leaf segments of leaf-1 for palms DR2, DR21 and DR25 during the first 70 days *in vitro* are shown in Fig. 1. After 24 h *in vitro* the mitotic index fell significantly from 1.3 ± 0.3 to $0.5 \pm 0.15\%$ ($t = 2.35$; $p = 0.02$–0.05). It remained at this level during the first week, but dropped further to about 0.1% by day 14, rising somewhat at day 28 but was almost zero on day 70 (Fig. 1).

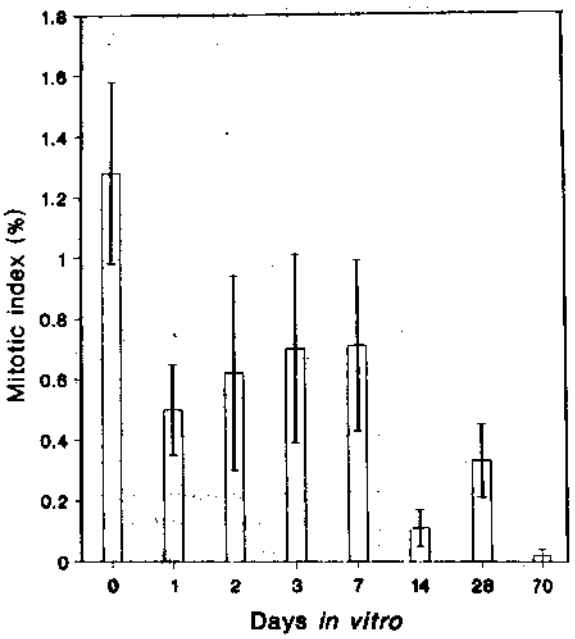

Fig. 1. The relationship between the mitotic index (±S.E. of the mean) and time in culture (days) for basal leaf segments of palms DR2, DR21 and DR25 cultured on Y3 medium ($n = 5$).

Microdensitometry

In the basal leaf segments of palm A, 89% of the cells were in the G1 phase of the cell cycle (Fig. 2). The histograms indicate that few cells were in S-phase; the majority were in G1. There was no significant difference between the distribution of nuclear DNA contents of cells in leaf segment 1 compared with segment 2 as shown by a two-tailed Mann-Whitney U test ($p = 0.12$). The corresponding non-parametric test between segments 1 and 3, and 2 and 3, showed significant differences ($p < 0.001$). These highly significant differences were the result of the skewing of the peak for G1 cells from 2.0–2.2 C for segments 1 and 2 (Fig. 2a-b), to 2.2–2.6 C for segment 3 (Fig. 2c). Nevertheless, we suggest that the distribution of DNA amounts in segment 3 is consistent with the majority of cells being in G1 of

Table 2. Summary of degrees of freedom (df), mean square (ms), F-ratio (F) and the significance levels in a series of two-way analyses of variance for the mitotic index data from the basal leaf segments of (*a*) leaf-1, palms A, B and C; (*b*) leaf-2, palms 1,2 and 3.

Source of variation	df	ms	F
(*a*) Palms A, B and C segments 1–3			
Leaf segment	2	0.08541	0.428 NS
Palm	2	2.3990	12.155 * * *
Leaf segment × palm	4	0.1004	0.509 NS
(*b*) Palms 1,2 and 3 segments 1–5			
Leaf segment	4	0.2965	1.735 NS
Palm	2	1.5606	9.135 * * *
Leaf segment × palm	8	0.2166	1.268 NS

Significance levels: * * * $p < 0.001$, NS-not significant.

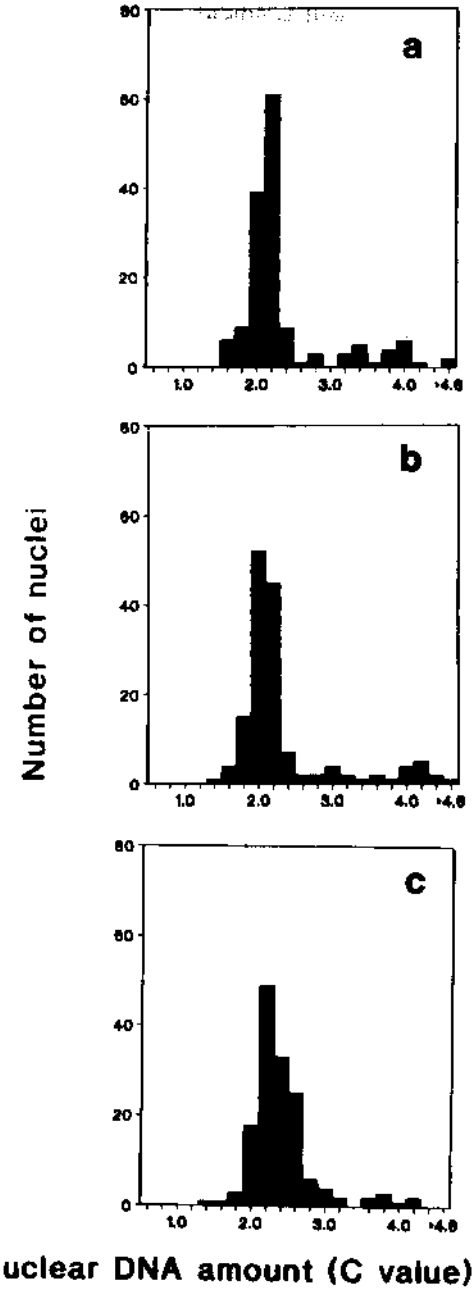

Fig. 2. The distribution of nuclear DNA contents (C value) of interphase nuclei from squash preparations of basipetal leaf segments: 1 (*a*), 2 (*b*) and 3 (*c*) of leaf-1 from palm A ($n = 150$ per histogram).

the cell cycle. Moreover there were no significant differences revealed by the Mann-Whitney U test on the corresponding DNA distributions for the corresponding segments in palms B and C (data not shown, associated probabilities = 0.5–0.9).

For reasons outlined in the Materials and methods microdensitometric measurements on *in vitro* samples were carried out on sectioned cells. Although not ideal for microdensitometry [16], the data from the sectioned material indicated that prior to culture about 90% of leaf cells of palms DR2, DR21 and DR25 were in G1 (Fig. 3; Table 3). This value (eg. Fig. 3a) is virtually the same as that obtained from squash preparations of leaf segments taken from palms A (Fig. 2a), B and C [14]. On the whole, this distribution of nuclear DNA amounts was maintained on days 14 and 28 *in vitro*. However, the detection of <1.6C DNA amounts, notably at day 28 for both DR25 and DR2, is consistent with nuclear breakdown and, hence, cytological instability. On day 70, between 20 and 40% of cells were in S-phase with a concomitant reduction in the percentage of cells in G1 (Table 3). The broadening of the DNA C value histogram (Fig. 3d) may be related to the re-initiation of growth in these cultures at this time.

During the culture period cells remained diploid, apart for one cell having a 6.2C nuclear DNA amount (Palm DR2, day 70; Table 3). Other cells classified as polyploid had nuclear DNA amounts of 4.6C and were part of the normal distribution of 4C nuclei (J.H.F. Jesty & D. Francis, unpublished data). Thus, there was little indication of the occurrence of polyploidy during the culture of the leaf explants.

Cell and nuclear area

Preliminary work established that a minimum of three replicates of 30 measurements per slide and, hence 90 measurements per sample, was sufficient to represent the true population mean [14]. There was no significant difference between mitotic cell and interphase cell areas *in vivo* (Table 4; $t = 1.53$; $p > 0.05$). However prophase nuclei were significantly larger than interphase nuclei (Table 4, e.g. $t = 9.33$; $p < 0.001$). Whilst there were fluctuations in cell and nuclear size from segment to segment, and from palm to palm, all interphase cell and nuclear areas were within the approximate range, 200 to 240 and 40 to 55 μm^2, respectively. Although, in general, cell size was larger in cells from leaf segment 1

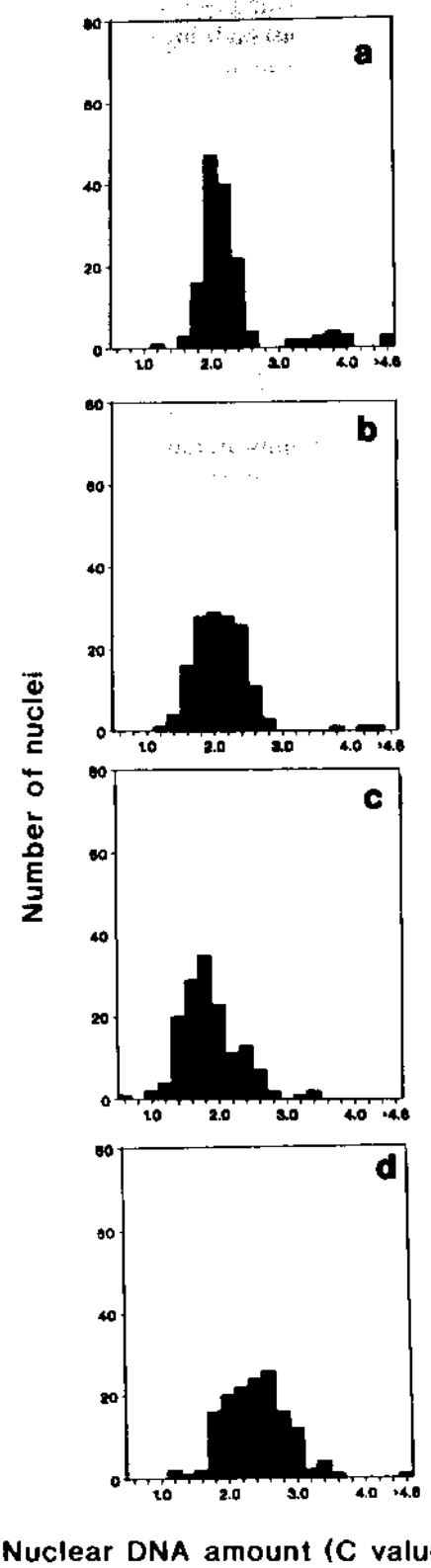

Fig. 3. The distribution of nuclear DNA contents (C value) of interphase nuclei from sections of basal leaf segments *in vitro* taken from leaf-1 of palm DR2 on (*a*) 0, (*b*) 7, (*c*) 14 and (*d*) 28 days following the start of culture ($n = 150$ per histogram).

compared with leaf segment 2, the nuclear size was also larger (Table 4). Thus, the nuclear cytoplasmic ratios for coconut leaf tissues *in vivo* are fixed.

Both epidermal and mesophyll cell areas increased after 1 day *in vitro* (Table 5; $p < 0.001$). The percentage increase in cell size was about 20 to 30% for mesophyll cells and 8 to 30% for epidermal cells. The nuclear area remained fairly constant over the culture period for both cell types. The overall consistency of nuclear size resulted in a drop in the nuclear:cell area ratio of mesophyll cells by day 1 after which there was no further reduction whilst a drop in this ratio for the epidermal cells did not occur until day 2 (Table 5). In general, the data are consistent with rapid increases in cell area and fluctuations in nuclear area upon transfer of leaf explants to culture media. In other words, nuclear and cell size changed independently when transferred from the *in vivo* to the *in vitro* environment. Note that the *in vivo* measurements of cell and nuclear size were larger than those made on the *in vitro* material. This is because the former were from unsquashed cell monolayers regardless of tissue origin whilst the latter were from tissue-specific sectioned material.

General observations on leaf explants transferred to culture media

Within the first week of explant initiation some leaf segments had visibly expanded, sometimes to several times their original size. This was most marked in the explants cultured in a liquid medium, 4A, comprising the same components as Y3 but modified as follows: 2,4-D (2.5×10^{-4} M) plus 6- (, -dimethylallylamino) purine (10^{-5} M) and activated charcoal (0.25%). Browning of the tissue (cut or wounds), particularly around the periphery of the explant, was most evident in explants cultured on the Y3 medium. In some cases up to 80% of these explants showed browning after only one week

Table 3. Percentage of interphase cell with nuclear DNA C values of <1.6, 1.6–2.4 (G1), 2.4–3.6 (S), 3.6–4.8 (G2) and >4.8 C (polyploid), in basal leaf segments of palms DR25, DR21 and DR2, cultured on Y3 medium on days 0, 14, 28 and 70 *in vitro*.

Days *in vitro*	C values				
	<1.6	1.6–2.4 G1	2.4–3.6 S	3.6–4.8 G2	>4.8
DR25					
0	0.7 ± 0.7	89.3 ± 2.4	7.3 ± 1.8	2.7 ± 1.8	0
14	12.7 ± 5.9	84.0 ± 4.6	2.0 ± 1.1	0.7 ± 0.7	0.7 ± 0.7
28	51.3 ± 7.4	46.0 ± 8.0	2.0 ± 1.1	0.7 ± 0.7	0
70	8.7 ± 5.9	47.6 ± 2.2	41.0 ± 4.9	2.7 ± 1.8	0
DR21					
0	2.7 ± 1.3	85.3 ± 1.8	6.7 ± 1.3	4.7 ± 0.7	0.7 ± 0.7
14	14.7 ± 13.7	80.7 ± 12.2	2.7 ± 2.7	2.0 ± 2.0	0
28	3.3 ± 3.3	89.3 ± 6.7	6.7 ± 2.9	0.7 ± 0.7	0
70	8.7 ± 4.4	71.3 ± 8.7	19.3 ± 12.3	0.7 ± 0.7	0
DR2					
0	0.7 ± 0.7	85.3 ± 2.9	5.3 ± 1.8	6.7 ± 1.3	2.0 ± 1.1
14	3.4 ± 3.4	85.3 ± 4.0	9.3 ± 4.7	2.0 ± 1.1	0
28	18.0 ± 9.9	74.0 ± 10.4	8.0 ± 1.1	0	0
70	2.0 ± 12	56.4 ± 8.3	40.0 ± 9.3	0.7 ± 0.7	0.7 ± 0.7

Table 4. Mean nuclear and cell area (μm^2 ± S.E. of the mean) and nuclear (N): cell (C) are ratios recorded from unsquashed cell monolayers for (*a*) cells in interphase, (*b*) mean cell area for mitotic cells, and (*c*) mean nuclear area for prophase cells in basal leaf segments of leaf-1 dissected basipetally from palms A, B and C.

Palm	Segment	Cell area	Nuclear area	N:C
(*a*) Interphase cells				
A	2	240.3 ± 11.9	45.2 ± 1.0	0.187
B	1	229.2 ± 7.3	53.7 ± 1.2	0.230
B	2	200.1 ± 6.8	45.7 ± 1.1	0.225
C	1	228.0 ± 8.2	51.9 ± 1.2	0.223
C	2	199.8 ± 9.7	40.8 ± 1.3	0.200
(*b*) Mitotic cells				
B	1	211.4 ± 9.0	–	
B	2	256.4 ± 7.6	–	
C	1	232.5 ± 9.6	–	
(*c*) Prophase cells				
A	1	–	72.9 ± 2.1	
B	1	–	68.9 ± 1.1	
B	2	–	58.8 ± 1.0	
C	2	–	68.3 ± 1.3	

in vitro. The rate of expansion of leaf explants was not dependent on their origin within the leaf i.e distance from the leaf base (data not shown). By day 28 on Y3, the lamina had undergone considerable expansion of both the mesophyll and epidermal cells (Fig. 4). However, in some regions of the explant there was a sharp demarcation between small cells, which resembled original leaf cells and large expanded cells. This morphology was maintained throughout the first month *in vitro* by which time expanded cells were observed many of which lacked nuclei. In

Table 5. Mean nuclear and cell area ($\mu m^2 \pm$S.E. of the mean) and nuclear (N): cell (C) area ratios recorded from sections of epidermal and mesophyll cells in basal leaf segments of the immature coconut leaf cultured on Y3 medium on days 0, 1 and 2 following culture initiation.

Days *in vitro*	Cell	Nuclear	N:C
Epidermis			
0	118.6 ± 3.3	15.3 ± 0.3	0.129
1	135.6 ± 4.2	17.5 ± 0.4	0.129
2	151.9 ± 4.3	18.6 ± 0.4	0.122
Mesophyll			
0	344.0 ± 13.7	20.1 ± 0.4	0.060
1	430.6 ± 19.1	17.4 ± 0.7	0.040
2	441.9 ± 17.5	19.4 ± 0.5	0.040

contrast, unexpanded regions comprising intact cells and nuclei existed in which the tissue continued to resemble leaf tissue (Fig. 4). The majority of mitotic cells were confined to the unexpanded regions of the explant. These observations indicate that although considerable cell expansion had occurred, cells were still capable of division and growth in the unexpanded regions of explanted leaf tissue. Although callus was initiated from these leaf explants none of them become morphogenetic.

Fig. 4. Transverse section of a coconut leaf explant after 28 days in culture showing a clear junction between unexpanded and expanded vacuolated cells (×550).

Competence of cells to respond *in vitro* is related to the developmental age of the explant, and this is clearly demonstrated in the developing gramineous leaf [17, 18]. For example, in *Lolium multiflorum*, a developmental gradient exists within the fourth foliage leaf [9]. Callus formation was associated with the meristematic state of the leaf tissue. Callusing frequency also depends on the spatial and temporal developmental state of the explant (see Introduction). Establishing the cell cycle status of the leaf tissue prior to culture, and whether a developmental gradient exists within the base of the immature coconut leaf was therefore the aim of the work reported here.

Conclusions

In basal leaf segments from leaf-1 and leaf-2, the mitotic index ranged from about 0.5 to 2% demonstrating a complete absence of a gradient of cell division (Fig. 1). These data are in remarkable contrast with the striking basipetal gradient of cell division observed in the second foliage leaf of various wheat species [6, 19] and in the fourth leaf of *Lolium temulentum* [20]. An explanation for the differences between coconut and these other monocots may lie in the two separate modes of leaf development in coconut and wheat. The compound leaf of the palm is characterised by the development of distinctive pleats, or plications [11]. The leaf primordium develops as a hood over the apex. The leaflets appear as a series of ripples on the surface of the blade and then, the pleats separate into individual leaflets. Clearly, the development of the gramineous leaf is quite different beginning with apical divisions followed by the activity of several meristematic regions which contribute to further development, notably the intercalary meristem. As the leaf ages, the intercalary meristem ceases activity and cells differentiate [9, 21]. In the coconut primordium, meristematic activity is restricted to the ground meristem [22]. As the palm leaf blade

develops, meristematic activity remains localised in the middle layers of the lamina [23]. The difference in the morphogenesis of the coconut and wheat leaves has a bearing on the morphogenetic potential of these tissues as explants because whilst the former produce only callus *in vitro* the latter can form roots and shoots [see Introduction].

The majority of cells in the immature coconut leaf are in G1 of the cell cycle (Fig. 2). However, the low mitotic indices suggest that a small percentage of cells were cycling (Fig. 1) and microdensitometry revealed that at least 6% of cells were in S-phase. Thus, selective accumulation of cells in G1 occurs both *in vivo* and *in vitro*; as yet there is no ideal *in vitro* mileau capable of altering this cell cycle pattern (see Introduction). Clearly, factors that could stimulate these cells to undergo this G1/S transition may well result in an increase in the proportion of cycling cells, faster growth rates and, perhaps, increased morphogenetic potential.

In coconut, there was no difference between mitotic and interphase cell area, but nuclear area was larger in prophase cells (Table 5). Nuclear size is not correlated with nuclear DNA content [24, 25] and in some species (e.g. *Vicia faba*) nuclear size is subject to considerably variability [26, 27]. In coconut leaves, any change in cell size was accompanied by a proportionate change in nuclear size (Table 4). However, following explant initiation, cell size in both epidermal and mesophyll cells increased within the first 24 h by 20–30 and 8–30%, respectively (Table 5). The rise in mesophyll cell area, however, was not accompanied by a proportional increase in nuclear area. Hence, there was a fall in the nuclear:cell area ratio which did not alter thereafter. However, for the epidermal cells this ratio fell on day 2. Clearly, the transfer of coconut cells from an *in vivo* to an *in vitro* environment disrupted the co-ordinated relationship between cell and nuclear size. This was also noted for *V. faba* when germinating roots were transferred from vermiculite/perlite or sand to water [27]. Moreover, when roots of *V. faba* are excised and transferred to culture media cell size increased, and nuclear size fell [7]. These observations demonstrate that cell and nuclear size alter independently of each other when cells are transferred from an *in vivo* to an *in vitro* environment. The data reported here and elsewhere [12] indicate that the regulation of cell and nuclear parameters in coconut are under separate control. Clearly, the lack of growth and regenerative ability of coconut cells *in vitro* must relate, in part, to both an accumulation of cells in G1 and uncontrolled cell expansion. Gould [28] argued that under such conditions factors necessary for cell division become diluted below a threshold capable of re-initiating cell division. The data reported here support that view. Other markers of recalcitrance *in vitro* have been found for coconut by comparisons with hexaploid wheat as a model system, and by using iso-enzymes as markers of proliferative/organogenetic potential. These data will be the subject of future communications on the cellular behaviour of coconut cells *in vitro*.

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