

Oil palm *in vitro* regeneration: microdensitometric analysis during reproduction and development

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Abstract

An extensive microdensitometric analysis was performed in oil palm *Elaeis guineensis* Jacq. during the establishment of a new embryogenic culture, on new regenerated plants at different times, and finally on seedlings obtained from adult regenerated normal and abnormal plants. Moreover nuclear DNA content was determined as well during the progression of meiosis. A variable loss of DNA content/nucleus in regenerated plants was demonstrated, in some cases very severe, depending mainly on the *in vitro* stationing of explants before the regeneration induction; DNA variation, but to less extent, was recorded also in seedlings from adult mother plants of the same genotype, *in vivo* and *in vitro* propagated. Considering normal and abnormal regenerated plants, DNA content variations did not seem to be necessarily connected to the abnormal phenotype. In addition stable and different variants in genome size could be obtained from *in vitro* culture of the same explant. Changes in the values of nuclear DNA content were determined during the meiotic prophase, confirming DNA extrusion processes also observed in other plant systems *in vivo*. Indeed the regeneration process may induce a quantitative DNA modulation/loss per cell in the regenerated plants but progressively DNA sequences should be regained before completion of the meiotic process. This information points to mechanisms of somaclonal variation.

Keywords: *Elaeis guineensis* Jacq, quantitative DNA modulation, microdensitometry, regeneration system, somaclonal variation, meiosis

Introduction

In vitro culture of oil palm, *Elaeis guineensis* Jacq., is generally used for the large scale propagation of this subtropical plant, the most productive oil-bearing crop in the world. Oil palm is largest source of vegetable oil used in the food sector (high quality oil and its derivatives) and in industrial activity (biofuel) (Sumathi et al. 2008; US Department of Agriculture 2015). The oil palm tree can reach a height of 20 m or more at maturity and, being a monoecious crop, male and female inflorescences are present in different parts of the same plant. Four oil palm varieties have been distinguished on the basis of the fruit structure, especially the thickness of the endocarp and consequently oil production: *E. guineensis* var. *macrocarpa* with 40–60% shell, *E. guineensis* var. *dura* with 20–40% shell, *E. guineensis* var. *tenera* with 5–20% shell and *E. guineensis* var. *pisifera*, a shell-less form. Inbreeding studies have demonstrated that *E. guineensis* Jacq. var. *tenera*, a hybrid obtained from var. *dura* × var. *pisifera* (D×P), gave the best quality fruits (bunches of drupes) with the highest palm oil (Sambandamurthi et al. 2009). For that reason selected F₁ plants of var. *tenera* represent the initial biological material to obtain *in vitro* cultures and to regenerate somaclones with the same characteristics of the mother donor plants used to establish new oil palm plantations. Like all plant systems replicated *in vitro*, clonally propagated oil palm plants can manifest morphological changes at genetic and epigenetic level during the different developmental stages. This phenomenon is termed somaclonal variation and it is due to cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, alteration in DNA methylation, gene activation and silencing (Nuti Ronchi et al. 1992a, 1992b; Nuti Ronchi 1995; Geri et al. 1999; Kaeppler et al. 2000; Jain 2001; Bairu et al. 2011).

Unfortunately, among regenerated oil palms, somaclonal variants with affected reproductive organs constitute at least 5% of regenerated plants and they can be recognized only at maturity, after 5–6 years from implantation, causing big yield and economic loss in palm cultivated areas. In particular, the somaclonal variation known as “mantled” (Corley et al. 1986) results in anomalous fruit formation for the proliferation of woody supernumerary extracarpels originated from normally abortive stamens of the female flowers.

Previous studies on the *in vitro* regeneration system of oil palm established the importance of the cultural parameters in reducing the mantled incidence on regenerated plants (Besse et al. 1992; Duval et al. 1995; Mgbeze and Iserhienrhien 2014).

By means of flow cytometric analyses of different types of calli (embryogenic and non embryogenic) and plants obtained from the cross *dura* × *pisifera*, it was demonstrated that embryogenic calli and plants showed the same ploidy level, but the measured 2C DNA values differed significantly (Rival et al. 1997).

Moreover, previous studies on the *in vitro* regeneration system of oil palm established the relationship between genomic DNA hypomethylation and mantled somaclonal variants, confirming the epigenetic nature of the mantled phenotype (Jaligot et al. 2000). More recently, by means of transcriptome studies, Shearman et al. (2013) analysed the expression profiles of normal and mantled late developing female flowers. The authors concluded that the mantled phenotype was function of a non-specific disruption of the methylation pathway, induced by somatic embryogenesis and by the activation of different gene expression patterns. In other plant systems, by means of

cytogenetic and molecular approaches, it was demonstrated that primary explants always underwent processes mimicking sexual maturation, included somatic meiosis and floral primordia differentiation (Giorgetti et al. 1995; Geri et al. 1999; Pitto et al. 2001). These phenomena arose in somatic cultured cells very early, following a genetic reprogramming plan in which committed cells could acquire a gamete-like condition, erasing the previous information related to the somatic differentiated status and getting a committed totipotent status. In particular, in carrot somatic embryogenesis, microdensitometric analysis demonstrated the modulation of nuclear DNA content during the establishment of the culture and the different phases of somatic embryogenesis. The DNA loss was exemplified mainly by unique sequences, and was recovered during development (Geri et al. 1999).

Analogous results were obtained in oil palm, by measuring nuclear DNA content in zygotic embryos during germination and during the constitution of embryogenic cultures (Giorgetti et al. 2007). Moreover, the study of meiosis by immunocytochemistry analyses showed the presence of DNA extrusion of methylated heterochromatic sequences, validating the idea of DNA content modulation during the development *in vivo* and *in vitro* (Giorgetti et al. 2007).

In this work, by means of microdensitometric analyses, the DNA content of regenerated plants at different times of development, before and after the transferring into the field, the DNA content of seedlings (obtained from seeds derived from adult regenerated normal plants and abnormal mantled plants) and finally the DNA content during the progression of meiosis, are analysed in order to assess the possible genomic instability related to the genome size in the different systems.

Materials and methods

Plant materials

Root apices of oil palm plants of different genotype and origins were analysed for DNA content determination by microdensitometric analyses:

(1) Root apices of oil palm seedlings regenerated *in vitro* from embryogenic calli (callus A; callus B; callus C; callus D). Oil palm calli were obtained from *in vitro* culture of frond explants of *E. guineensis* var. *dura* × *pisifera* (DxP); the different calli were maintained for 24 months in MS medium (Murashige and Skoog 1962) with addition of 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 1% gelrite, and monthly sub-cultured. Embryogenesis was induced after the hormone removal. Regenerated plantlets were grown in sterile pots for a total period of about two months; at this time roots were well developed and microdensitometric analysis of root apices was performed.

(2) Root apices of oil palm plantlets *in vitro* regenerated from zygotic embryos cultures 309/125 DxP genotype. For the establishment of fast growing embryogenic *in vitro* cultures, zygotic embryos were isolated in sterile conditions from dry seeds and put in MS solid medium with the addition of 1 mg l⁻¹ 2,4-D. After two months embryogenic calli started to develop somatic embryos that were isolated and transferred in pots. Root apices were analysed in two-monthold plantlets.

(3) Root apices of 6 DxP regenerated plants (derived from *in vitro* culture of a single frond explant) transferred in field (DxP ramets).

(4) Open pollination derived seedlings (ortets) from L27 (DxP) mother plant and normal/abnormal ramets from the same genotype.

(5) Oil palm pollen mother cells from L27 (DxP) plants at first and late prophase of meiosis I.

All the oil palm material was kindly provided by the Malaysia Palm Oil Board.

Microdensitometric analysis

The Feulgen-DNA absorption cytophotometric analysis offers the possibility to determine the cellular DNA content in different biological systems. Microdensitometric analysis is slower and much more laborious in comparison with flow cytometry, but it offers various advantages. Measurements can be made on individually selected cells, and the slides provide a permanent record and can be retrieved for future studies. In addition microdensitometry allows the analysis of every cell population at the same time, independently of the size of the measured nuclei, strongly reducing the risk to ignore nuclear events present in low number. Therefore this technique, unlike flow cytometry, enables a complete view of the entire tissue cell population, giving a picture of the dynamics involved along the culture process. Moreover the advances in equipment, including the use of computers, in part simplify the measuring procedure, and further expand the potentials of the technique.

In this work by means of microdensitometric analysis of root meristems, the DNA content of oil palm plants of different origin, genotypes and at different developmental stages was determined.

For cytophotometric analysis, five root tips from regenerated and control plants were fixed in 4% formalin, thoroughly washed in running tap water and squashed, after pectinase-cellulase treatment (pectinase 20% cellulase 5% for 45 minutes) under a cover slip in a drop of 45% acetic acid. Slides were frozen with dry ice, and the coverslips removed. Samples were hydrolysed in 5 N HCl at room temperature for 60 min, stained with Schiff's reagent for 45 min and washed in fresh 4% sodium meta-bisulphite (three times for 5 min) prior to dehydration and mounting (Giorgetti et al. 1995).

Feulgen/DNA absorption in individual nuclei was measured with the two-wavelength method (542 and 498 nm) by a MPV (Mikrophotometer mit Variable) Compact Leitz microscope photometer equipped with a mirror scanner and interfaced to a PC with a specific software. Alternatively microdensitometric analyses were done with a light microscope with a dedicated macro for microdensitometry image analysis, Zeiss Axio Vision 4.6: Carl Zeiss Microscopy GmbH, Germany.

Before starting our analysis utilizing this second system, we established the validity of the software by comparing the quantitative analysis obtained on root tip apices of *Vicia faba* and *Allium cepa*, firstly measuring with the two-wavelength method using an MPV Compact Leitz microscope-photometer and then with the Zeiss Axio Vision program on the light microscope. The results obtained with the two systems were perfectly equivalent and after normalization were expressed as arbitrary units (AU). As a reference we used human lymphocyte preparations (2C = 6 pg), *Vicia faba* L. (2C = 26 pg) and *Allium cepa* L. (2C = 33.5 pg) (Bennett and Leitch 2005), and the calculation based on these standards gave a 2C DNA content of 3.7 pg, slightly lower than that determined by flow cytometry, showing a 2C content of 3.76 pg (Rival et al. 1997). Absorption values were obtained measuring 100 nuclei in three different preparations of the same sample (total of 300 nuclei).

Cytological analysis of pollen mother cells

Cytological analysis was performed in pollen mother cells after fixation in Carnoy's solution, ethanol:glacial acetic acid (3:1 v/v), for meiotic stage determination. Pollen mother cells were isolated from male flower anthers and stained following the Feulgen technique as previously described (Giorgetti et al. 1995, 2007). Once the meiotic stages were identified, a part of the anthers was used for the microdensitometric analysis following the protocol described above for root apices; five different anthers for every sample were analysed.

Statistical analysis

All statistical analyses used GraphPad (GraphPad Software, Inc., La Jolla, CA, USA, <http://www.graphpad.com/>) Prism version 6.0 performing ANOVA and Tukey post-hoc multiple comparisons test.

Results and discussion

In vitro seedlings from nodular calli

Microdensitometric analysis was first carried out on root apices of oil palm plantlets regenerated *in vitro* from old embryogenic nodular calli A–D derived from *in vitro* culture of DxP immature leaves and for comparison on root apices of *in vitro* regenerated plantlets derived from zygotic embryos (309/125 DxP genotype). The results are shown in Figure 1. A strong decrease of DNA content, expressed in arbitrary units (AU), was observed in the root tips belonging to regenerated plants in comparison to the control (DxP seedling). The lower DNA contents were recorded in root tips of plants regenerated from old calli, in particular callus A, in which the decrease reached 29.62% when compared with the 4C value of control plants.

The differences from the control were less marked for plants regenerated from newly established cultures from zygotic embryo (7.74% and 3.38% less for plantlets 1 and 2 respectively) but they were still of high statistical significance, as were all the other recorded differences.

Genomic disturbances are the basis of somaclonal variation that includes several categories of genomic perturbations, differing from species to species. These changes include, apart from chromosomal reorganization, point mutations and insertion of mobile DNA elements or retroelements, epigenetic alterations (Schellenbaum et al. 2008; Mujib et al. 2013), and rearrangements in nuclear or organellar DNA and alteration in ploidy level (Jain 2001) leading to variation in the genome size. The observed differences in DNA content indicate that the effects of *in vitro* culture and regeneration are more pronounced in plants derived from old calli obtained by leaf explants. Conversely, the callus production from zygotic embryos, being faster and foresee a shorter period of *in vitro* culture in respect to leaf explants (Giorgetti et al. 2011; Balzon et al. 2013), led to the propagation of high quality plants with less damaged DNA content.

DNA content analysis of oil palm regenerated plants derived from the same DxP explant

To extend the microdensitometric analysis concerning the *in vitro* regeneration process, six regenerated plants derived from the same DxP explant were examined after the transfer into the open field. Figure 2 summarizes the cytophotometric results, obtained from the analyses of the oil palm root meristems, expressed in AU. All the *in vitro* regenerated plants showed a decrease of DNA content/nucleus when compared to the 4C value of DxP

control plant (6.03 AU) ranging from 7% (ramet 5) to 25% (ramet 4). Tukey's multiple comparison analysis revealed that all the differences were statistically significant in comparison to the control but the 4C content in ramets (mean value 4.64 AU), with the exception of ramet 5 (5.53 AU), was not significantly changed.

These results clearly show that regeneration processes in oil palm trigger phenomena of genomic rearrangements that lead to a reduction in DNA content, able to regenerate, from the same explant, different stable variants in genome size. The occurrence of genome size variants may be a consequence of various and different cellular response to *in vitro* culture stress, likely leading to the expression of altered phenotypes during plant growth and development.

DNA content analysis in ortet and ramet plants

An extensive microdensitometric analysis was carried out on 10 different oil palm seedlings derived from the L27 DxP genotype. In this case the analysed seedlings derived from mature plants which could have a different origin, from seeds (ortets) or from *in vitro* regeneration (ramets). In particular the analysis was conducted on four seedlings derived from open pollination seeds obtained from L27 ortet plant, three seedlings from L27 normal ramet, and three seedlings from L27 abnormal ramet plant. The analysis made it possible to compare the DNA content of seedlings coming from the same genotype but derived from different adult plants grown in the open field until fruit/seed production, obtained by both *in vivo* and *in vitro* propagation approaches. The results are illustrated in the histogram of Figure 3. Also in this system variations in 4C DNA content were observed, lower than the cases already described; however DNA content variations did not seem to be connected to the *in vitro* induction and to normal and abnormal phenotypes. Since, normally, oil palm plants take some years before producing seeds, irrespective of whether that they have originated *in vivo* or *in vitro*, the stationing in the open field until flowering and seed production, after meiotic process and embryo formation, could reduce the recorded variation between the different seedlings. Although DNA content variation is not predictable in the same plant progeny, environmentally induced and/or developmentally regulated "genetic make-up" can be hypothesized, leading to a partial rebalancing in the genome size (Li 2009). However, *E. guineensis* possesses an intrinsic genomic instability probably due to its tetraploid origin, recently confirmed by molecular studies (Singh et al. 2013), and to its hybrid condition (Hartley 1988; Rayburn et al. 1993), both likely guiding genome size deviations. These changes in genome size were extensively recorded in our analyses both in plants regenerated from the same explants and in plants obtained from different genotypes and propagation methods.

Intraspecific variations in DNA content of *E. guineensis* have also been documented by Madon et al. (2008) by means of flow cytometry studies, stressing the importance of variations in genome size in influencing the phenotypic characteristics at the cellular and organism level and in providing important information on the genome evolution. The polyploid condition can enable the loss of a certain amount of DNA to be withstood, as observed in other plant organisms, even if the molecular mechanisms that induce this genome changes are not clear (Liu and Wendel 2002). In wheat it has been demonstrated that sequence elimination, together with variations in DNA methylation patterns, is one of the major and immediate responses of the genome to wide allopolyploidy or hybridization, affecting a large fraction of the genome (Shaked et al. 2001). Some probable candidates involved in these phenomena may be repetitive sequences, retroelements, transposons, being able to

change both the genomic position and the copy number, even if it is not possible to rule out the involvement of other regions of the genome.

DNA content analysis during male meiosis in oil palm

Microdensitometric analysis was performed during male meiosis to evaluate the possible DNA content modulation during the meiotic process already observed in other plant systems (Giorgetti et al. 2007).

The aim of our investigation was to check the presence of the DNA extrusion mechanism previously observed at early meiotic prophase, a mechanism which could be responsible for DNA content variation. In fact our earlier data obtained in barley by means of cytophotometric measurements of the nuclear DNA content at different meiotic stages, revealed a highly significant decrease of nuclear DNA content during meiosis I starting from middle–late prophase (Giorgetti et al. 2007).

Microdensitometric analyses were performed on pollen mother cells at early and late prophase of meiosis in L27 ortet plant and in L27 normal ramet plant (Figure 4). A decrease of nuclear DNA content values was evident from early meiotic prophase both in ortet and in normal ramet. In L27 ortet the estimated mean value of 4C was $\cong 6.3$ AU for early meiotic prophase and $4C \cong 5.9$ AU for middle–late meiotic prophase with a mean values difference $\cong 6.35\%$. The same trend was found in L27 normal ramet in which the mean value of 4C was $\cong 6.14$ AU for early meiotic prophase and $4C \cong 5.6$ AU for middle–late meiotic prophase with a mean values difference $\cong 8.79\%$: Differences between the early and late prophase stage of meiosis were highly significant when analysed with Turkey's multiple comparison test. Conversely, the differences between 4C mean values of L27 ortet early prophase ($4C \cong 6.3$ AU) and of L27 normal ramet early prophase ($4C \cong 6.144$ AU), and 4C mean values of L27 ortet middle–late prophase ($4C \cong 5.9$ AU) and of L27 normal ramet middle–late prophase ($4C \cong 5.6$ AU), were not significant. Microdensitometric results were further confirmed by cytological observations of the meiotic process with the presence of chromatin body extrusion from the bulk DNA during early prophase (Figure 5).

Meiotic division on one hand assures genome stability and integrity over sexual life cycles, and on the other can generate genome variations in several ways and has been considered a major driving force for gene and genome evolution in nature (Cai and Xu 2007). From our results we can conclude that DNA modulation characterizes the first meiotic division, during the transition from early to middle–late prophases with a DNA loss greater than 8%. This fact confirms other similar finding both in plants and in animal systems (Jankowski et al. 2000; Bucholc et al. 2001; Bennetzen 2002; Devos et al. 2002; Bennetzen et al. 2005) and our previous results (Giorgetti et al. 2007) which described, in oil palm and in barley, the extrusion of heterochromatin bodies during early meiotic prophase and their high methylation level. DNA extrusion can be associated with meiotic correction process necessary to get rid of somatic multicopy DNA sequences and ensure a functional chromosome pairing and crossing-over in order to produce viable gametes. According to Li (2009) a developmental correction in genome size may occur in some species during their lifespan, leading to a rejuvenation status by DNA loss in early meiosis and to a subsequent regaining in DNA sequences in a sort of “genomic variation cycle” in which many other cellular and environmental events are involved.

Conclusions

In order to characterize the genome size and its possible variations in oil palm, *in vivo* and during *in vitro* cultivation of oil palm, a widespread microdensitometric analysis was performed considering different systems.

During the plant life cycle, several mechanisms of genomic perturbation, e.g. chromosomal reorganization, point mutations and insertion of mobile DNA elements or retroelements, epigenetic alterations and changes in repetitive sequences and in ploidy level, can occur; these may be involved in the phenomenon of genomic variation in oil palm. Microdensitometric analyses demonstrated variable values of DNA content/nucleus in all the considered samples. The modulation of DNA content seems to be a recurring phenomenon in oil palm, at least in the different systems we have studied, both in normal conditions and *in vitro*. In natural conditions it is widespread in this species, probably being influenced by its tetraploid origin and hybrid condition. In particular along the meiotic process DNA loss mechanisms may be involved in the resection of somatic heterochromatin in the course of noncrossover events, in order to have perfect pairing and crossover products.

From these results it is clear that *E. guineensis* possesses a potential intrinsic genomic instability in genome size. The stress from *in vitro* culture on cellular behaviour amplifies natural genomic instability of *E. guineensis* becoming a real genomic disturbance leading to somaclonal variation.

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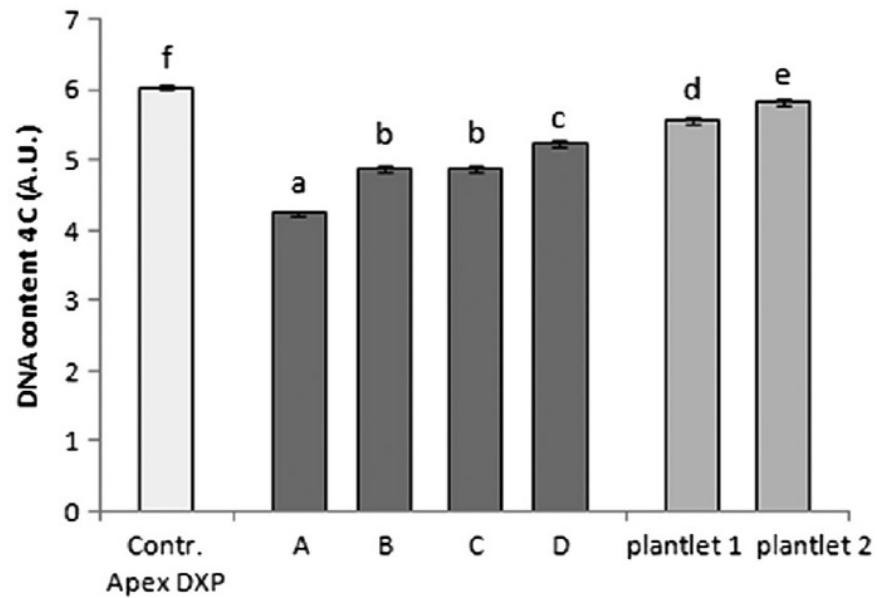


Figure 1. Microdensitometric analysis of root apices of oil palm seedlings regenerated *in vitro* from old embryogenic nodular calli A, B, C, D and for comparison on root apices of *in vivo* (Contr. Apex DXP) and *in vitro* regenerated plantlets (1 and 2) derived from zygotic embryos (309/125 DXP genotype). Different letters show significant differences among oil palm plants according to Tukey's test. Bar = standard error.

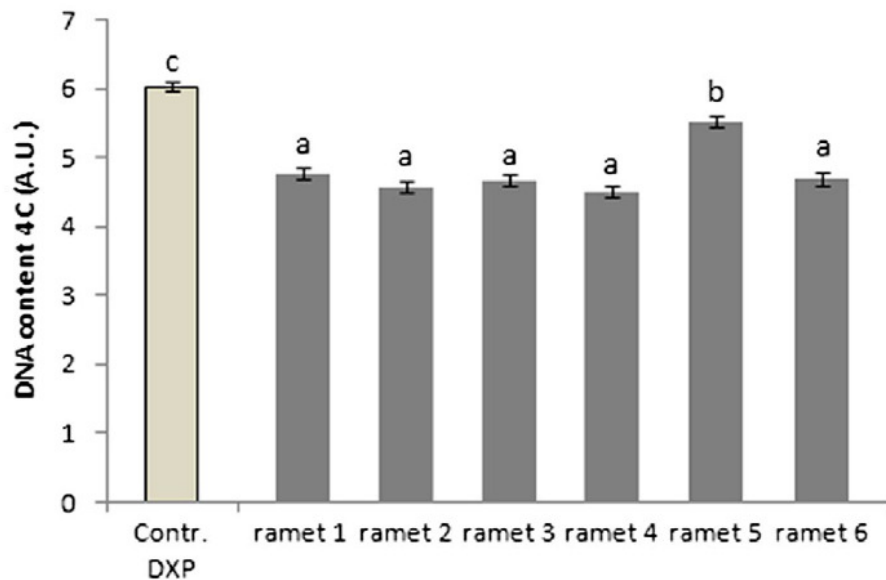


Figure 2. Cytophotometric measurements of the nuclear DNA content, expressed in AU (arbitrary units) of six different regenerated plants derived from the same frond excised from DXP oil palm plant. Different letters show significant differences among oil palm plants according to Tukey's test. Bar = standard error.

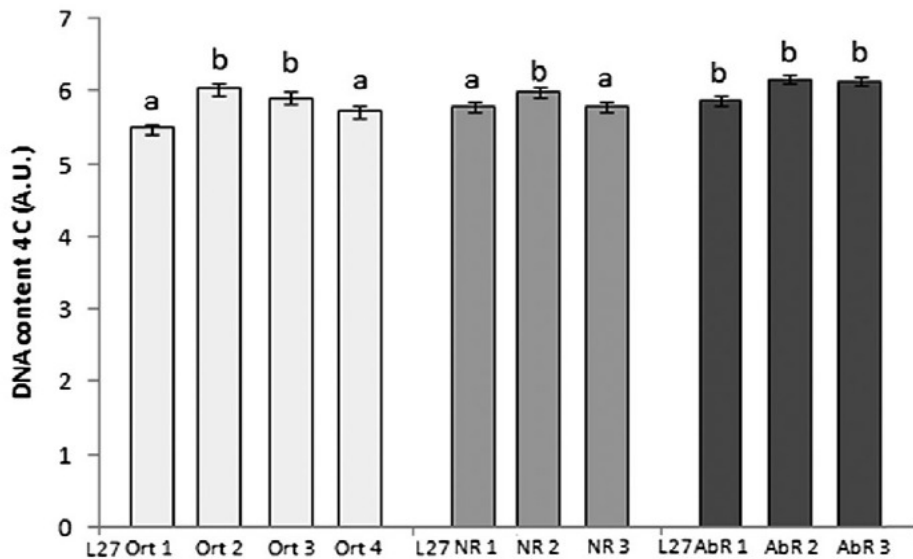


Figure 3. Cytophotometric measurements of the nuclear DNA content, expressed in AU (arbitrary units) in L27 oil palm seedlings: light grey columns show 4C values of seedlings derived from seeds of ortet plants, Ort); middle grey columns show 4C values of seedlings from seeds of normal regenerated plants (normal ramets, NR); dark grey columns show 4C values of seedlings from seeds of abnormal regenerated plants (abnormal ramets, AbR). Different letters show significant differences among oil palm plants according to Tukey's test. Bar = standard error.

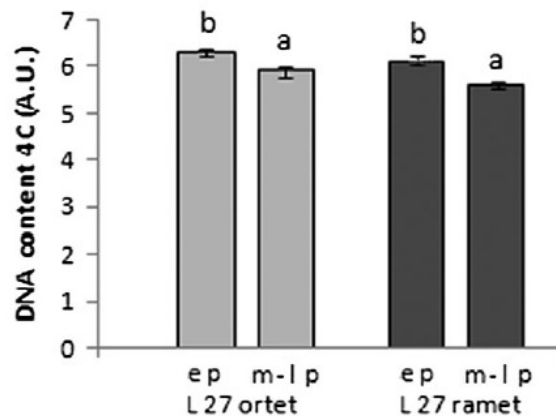


Figure 4. Cytophotometric measurements of the nuclear DNA content, expressed in AU (arbitrary units) of two different meiotic stages (early prophase, e p, and middle late prophase, m-l p) in oil palm Ortet L27 (grey) and in normal ramet L27 (dark grey). Bar = standard error.

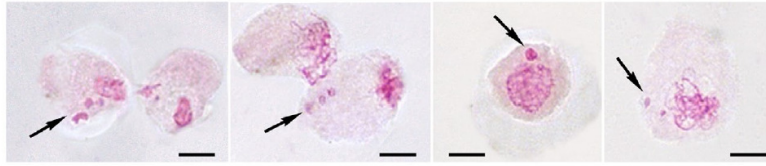


Figure 5. Pollen mother cells in *Elaeis guineensis* (L27 genotype) at prophase stage. Arrows indicate chromatin bodies out of the bulk DNA observed during cytological analysis after Feulgen staining. Scale bars, 5 μm .