Cytological Investigations on the Endosperm of *Borassus flabellifer* Linn.

J. Stephen

Department of Botany, University of Kerala,
Kariavattom, Trivandrum, India

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The endosperm serves as the main source of nutrition for the developing embryo and its failure results in the abortion of embryo and consequently low fertility. Economically, the tissue is of great importance since it is the store house of carbohydrates, proteins, lipids and growth regulating substances. Although much work has been done on the morphology and physiology of this tissue in a number of angiosperms, the record of cytological investigations on this is relatively meagre. The very interesting findings made on the cytology of coconut endosperm in this laboratory (Abraham 1963, Abraham and Mathew 1963, Abraham, Ninan and Gopinath 1965, 1966) gave inspiration to inquire into the cytological situations in the endosperm of allied genera. The observations made on the cytology of the endosperm in Palmyrah palm (*Borassus flabellifer* Linn.) are presented in this paper.

Material and methods

The endosperm for the present work was collected from palmyrah palms from a few localities in Trivandrum District. Endosperm at various stages of development was fixed in Carnoy's fluid in the noon hours and after a few changes of fixative acetocarmine smears were made.

The liquid endosperm found in the central cavity of the developing seeds was sucked out using hypodermic needle and to it was added Carnoy's fluid and then it was subjected to centrifugation at 250 rpm for two hours. The sediment was again fixed in Carnoy's fluid and acetocarmine smears were made.

In order to study the development of the endosperm 1 to 20 week old intact seeds were fixed in F.A.A. and microtome sections were made by the usual paraffin method. The slides were stained with iron haematoxylin and fast green.

To ascertain the chromosome number of *Borassus flabellifer* Linn. growing embryos from 4 to 5 month old nuts were fixed in Carnoy's fluid in the noon hours and acetocarmine smears were made.

Observations

The chromosome number of the species was ascertained as $2n (2x)=36$ (Fig. 1).

The earliest recognizable stage in endosperm development is the occurrence of

free nuclei in a mass of cytoplasm found towards the centre of the embryo sac cavity (Fig. A1). Sections of two week old endosperm show numerous free nuclei along with associated cytoplasm forming a lining layer close to the wall of the embryo

Fig. A. Camera lucida drawings of longitudinal microtome sections showing stages in the development of the endosperm in *Borassus flabelifer* Linn. 1, free nuclei distributed towards the centre of the embryo sac in one week old nut. $\times 70$. 2, initiation of the cellular state of endosperm in three week old nut. $\times 70$. 3, cellular endosperm in one month old nut. $\times 100$. 4, 3 month old cellular endosperm. $\times 70$. 5, cellular endosperm at the 4th month of development. $\times 70$.

sac. By the third week of endosperm development cell wall formation is initiated from the periphery towards the centre leading to the formation of cellular endosperm

(Fig. A2). Even after the attainment of cellular state, mitoses continue throughout the tissue (Figs. A3 and A4). To begin with, the cellular endosperm is more abundant at the chalazal region and progressively less so towards the micropyle. But by the fourth month the endosperm at the micropylar region also attains the same thickness as at the chalazal region whereas the remaining portions appear thinner.

During early stages of development the nuclei are of uniform size throughout the endosperm. As the tissue matures, along with these nuclei larger nuclei also appear. The average diameter of the nuclei is seen to increase with increasing maturity of the endosperm. The diameter of nuclei in the chalazal region is also found to be greater than that in other regions as data in Table 1 reveal. In sections of four month old endosperm three zones can be recognized on the basis of cell size (Fig. A5). The zone close to the seed coat is 5 to 10 cell thick and show cells and nuclei of almost uniform size. Measurements of nuclei and chromosome counts show that these are triploid in constitution. The region just inside this show variation in thickness in different regions of the endosperm. At the micropylar and chalazal parts this region is seen to be 50 to 60 cells thick, whereas it is only about 20 cell thick in the other parts of the endosperm. This zone is characterised by the occurrence of polyploid cells of larger size interspersed with predominantly triploid cells which are of the same size as those in the first region. The innermost zone is 1 to 5 cell thick throughout. This zone which borders the liquid endosperm is characterised by much larger spherical cells with abundant cytoplasm and nuclei which are similar in size to those of the first zone. Chromosome counts in this region could not be made as no cell division was observed in these cells. These cells are also characterised by the absence of prominent nucleoli. The centrifugate of the liquid endosperm at this stage shows in addition to the free nuclei certain differentiated cells (Fig. 21) identical with those of the innermost region just mentioned, whereas the centrifugate from younger nuts show only free nuclei. Abnormal giant nuclei with lobes and branches were observed both in the cellular and liquid parts of the endosperm of mature nuts (Fig. 19). By the end of the fifth month the endosperm fills the entire nut cavity and becomes very hard and cytological investigation becomes difficult. Figures suggesting amitosis were also observed in aged endosperm (Fig. 18).

Squash preparations of 1 to 4 month old solid endosperm showed mostly triploid cells with 54 chromosomes (Fig. 5) with a low frequency of cells showing various grades of ploidy ranging from 4x to 96x (Figs. 6, 8, 9, 10 and 11). With advancing maturity the number of cells with higher chromosome numbers showed steady increase as data in Table 2 reveal. Generally the polyploid cells showed chromosomes in multiples of the triploid number. Diplochromosomes have been observed both in triploid (Fig. 7) and highly polyploid nuclei. In one highly polyploid cell in addition to the diplochromosomes of usual size, numerous very minute diplochromosome like structures were also seen (Fig. 10). Diplochromosomes were also observed in the free nuclei of the centrifugate from the liquid endosperm (Fig. 20). In a few cells several daughter chromosomes were found to be held together at the kinetochore region to give the appearance of highly contracted

Figs. 1-9. Cytology of embryo and endosperm in *Borassus flabellifer* Linn. 1, somatic metaphase chromosomes from the embryo showing $2n$ ($2x$)=36. $\times 750$. 2-9. Cytology of the cellular endosperm. 2, triploid prophase with 3 nucleoli. $\times 500$. 3, triploid prophase with

Table 1. Distribution of endosperm nuclei of differing diameters in accordance with the age and the position in the developing seeds of *Borassus flabellifer* Linn.

Method	Age of endosperm	Size distribution of nuclei in the			
		Micropylar region		region half way between micropyle and chalaza	
		Range in diameter in μ	Average in μ	Range in diameter in μ	Average in μ
Microtomy	One month	16.125-19.35	18.473	16.125-19.35	18.32
	Two "	12.9 -19.35	19.26	12.9 -19.35	19.08
	Three "	12.9 -25.8	19.456	12.9 -22.575	19.14
	Four "	16.125-32.25	21.058	16.125-25.8	20.44
	Five "	16.125-32.25	22.24	16.125-32.25	21.79
Smear	One "	16.125-22.575	20.25	16.125-19.35	19.84
	Two "	16.125-25.8	22.45	16.125-25.8	20.83
	Three "	16.125-32.25	22.625	16.125-32.25	21.68
	Four "	16.125-32.25	22.86	16.125-32.25	21.865
	Five "	16.125-38.7	23.56	16.125-38.7	22.46
				Range in diameter in μ	Average in μ
				16.125-25.8	19.14
				16.125-25.8	21.3
				16.125-32.25	22.46
				16.125-32.25	23.54
				16.125-35.15	24.1
				16.125-29.025	21.36
				16.125-29.025	23.3
				16.125-32.25	23.97
				16.125-38.7	24.35
				16.125-45.15	26.05

2 nucleoli. $\times 500$. 4, triploid prophase with one nucleolus. $\times 500$. 5, triploid cell with 54 chromosomes. $\times 750$. 6, tetraploid cell with 72 chromosomes. $\times 750$. 7, c-mitosis in a triploid cell. $\times 750$. 8, hexaploid cell showing 108 chromosomes. $\times 750$. 9, a cell showing ca. 126 (7x) chromosomes. $\times 750$.

Figs. 10-18. Cytology of cellular endosperm. 10, a highly polyploid cell exhibiting c-mitosis. Note pulverization of chromosomes. $\times 750$. 11, a cell showing ca. 1728 (96x) chromosomes. $\times 190$. 12, a cell having 23 chromosomes. $\times 750$. 13, tripolar spindle at triploid anaphase. $\times 750$. 14, a cell showing highly contracted "chromosome bundles" in triploid number. Note that a few

chromosome bundles (Fig. 14). Except these chromosomes which were unusually thicker, the chromosomes of all cells with different grades of ploidy were of the same size. In addition to the euploid increase of chromosome number aneuploidy was also observed. Many of the aneuploid cells were hypotriploid (Fig. 12). Multipolar anaphases (Fig. 13) were encountered in one per cent of cells. At anaphase 0.9 per cent of the cells showed multiple bridges (Fig. 15). At telophase one per cent of the cells showed persistent bridges (Fig. 16).

The majority of $3n$ prophase showed 3 nucleoli (Fig. 2) while others had one or two nucleoli (Figs. 3 and 4). The number of nucleoli in hexaploid cells varied from 2 to 6. Both in triploid and hexaploid cells where the nucleoli were fewer in number, they were of larger sizes as data in Table 3 reveal. The nucleoli of mature endosperm cells showed prominent central vacuoles (Fig. 17).

Discussion

The somatic chromosome number ($2n=36$) of *Borassus flabellifer* Linn. observed in the present study is in conformity with the earlier reports (Venkata-subban 1948, Sharma and Sarkar 1956).

The development of the endosperm in *Borassus flabellifer* is of the nuclear type. After the free nuclear state, cell wall formation is initiated from the periphery and proceeds towards the centre leading to the formation of cellular endosperm as in *Cocos nucifera* (Abraham *et al.* 1966). However, Cutter, Wilson and Freeman (1955) are of the view that cellular endosperm in coconut is formed by the deposition of well-formed cells from the liquid part of the endosperm found in the central cavity. In *Borassus*, which is essentially similar to coconut in endosperm development, the centrifugate from very young nuts (1 to 3 month old) show only free nuclei. Whole cells are found only in the centrifugate from mature nuts. As these are identical with the large spherical cells of the central region of the cellular endosperm it is possible that these have been sloughed off from the cellular endosperm.

The endosperm is basically triploid in constitution with $3x=54$ chromosomes. Chromosome number in multiples of the $3x$ number have been found to be frequent. Diplochromosomes have been encountered at metaphase in cells having $3x$, $6x$ and $12x$ chromosomes and these presumably result from endoreduplication of chromosomes. Endoreduplication of chromosomes has never been observed at interphase of prophase in *Borassus*, though this has been found in other plants like *Zea mays*, *Tradescantia* sp. (Trad O_2) and *Chlorophytum laxum* studied by the author (unpublished). Levan and Hauschka (1953) have expressed the view that the configuration of diplochromosomes at metaphase is the result of endoreduplication. Many authors have used the term 'endomitosis' to describe similar events

chromosomes are not replicated. $\times 750$. 15, multiple bridge. $\times 750$. 16, persistent bridge. $\times 500$. 17, nucleus containing "ringshaped nucleolus". $\times 1500$. 18, nuclei showing amitotic tendency. $\times 300$. Figs. 19-21. Cytology of the liquid endosperm. 19, abnormal nucleus from the liquid endosperm of 4 month old nut. $\times 300$. 20, c-mitosis in a triploid free nucleus of the liquid endosperm. $\times 750$. 21, differentiated cells from the liquid endosperm of 4 month old nut. $\times 225$.

Table 2. Chromosome situation in the cellular endosperm of *Borassus flabellifer* Linn.

Maturity (in months)	Total no. of cells examined	Percentage of cells with										Aneuploidy		Percentage of polyploid cells above 3n
		3n	4n	6n	7n	9n	12n	24n	48n	96n	above 96n	Near 3n	Near 6n	
One	750	98.93	Nil	0.66	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0.4	Nil	0.66
Two	750	96	Nil	2.4	Nil	0.4	0.266	Nil	Nil	Nil	Nil	0.666	0.266	3.33
Three	750	93.2	0.533	3.33	0.266	0.666	0.133	0.266	0.266	0.133	Nil	0.8	0.4	5.993
Four	750	89.466	Nil	6.266	Nil	0.533	0.8	0.533	0.266	Nil	0.266	1.066	0.8	9.464

Table 3. The occurrence of nucleoli in relation to polidy status in the endosperm of *Borassus flabellifer* Linn.

Number of nucleoli	3n prophases				6n prophases				Percentage of occurrence out of 30 prophases
	Range in diameter in μ	Average diameter in μ	Percentage of occurrence out of 300 prophases	Number of nucleoli	Range in diameter in μ	Average diameter in μ	Percentage of occurrence out of 30 prophases	Number of nucleoli	
One	4.837 - 9.675	8.47	9.66	Two	8.02625 - 12.9	10.481	10		
Two	3.225 - 6.45	5.6547	24.34	Three	6.45 - 9.675	8.6	10		
Three	1.6125 - 4.8375	3.263	66	Four	3.225 - 9.675	6.181	15		
				Five	1.6125 - 6.45	3.7087	20		
				Six	1.6125 - 4.8375	2.926	45		

in the endosperm of a number of angiosperms (Geitler 1948, 1955, Steffen 1956, Jenschke 1959, Kapoor 1963, Nagl 1970a). The same phenomenon has been recorded in tapetum (Meyer 1925, Brown 1949) and suspensor (Jenschke 1959, Nagl 1962) of plants, salivary gland cells of diptera (Beermann 1952, White 1954) and in cancer cells (Levan and Hauschka 1953). In the endosperm of *Borassus flabellifer* Linn. as in *Cocos nucifera* (Abraham 1963, Abraham and Mathew 1963, Abraham *et al.* 1965, 1966) the type of endoreduplication is not endomitosis, but very much similar to c-mitosis, as there occurs the dissolution of nuclear membrane, condensation of chromosomes and even metakinesis. The inhibition seems to be at the level of the spindle mechanism. After replication the daughter chromosomes fall apart to give the appearance of ski-anaphase. But anaphase separation is prevented. As a result, a cell having double the original number of chromosomes is formed. The same process is repeated to give rise to higher ploid cells. Nagl (1970a, 1970b) is of opinion that the most general mechanism of polyploidization in the suspensor and endosperm of angiosperms is the "dispersion endomitosis" which is a prophasic event. However, the type of aberrant division by which endopolyploidy is brought about in the endosperm of *Borassus flabellifer* is a metaphasic event. Enzenberg (1961) has used the term "Restitutionkernbildung" (restitution nucleus formation) to describe the process by which endopolyploidy is achieved in the endosperm of 13 species of angiosperms she investigated. According to her "Restitutionkernbildung" occurs owing to the disturbances at the onset of mitotic anaphase. The type of aberrant division found in the endosperm of *Borassus* under the present study corresponds to "Restitutionkernbildung" described by Enzenberg (1961). The "chromosome bundles" observed in a few cells of the endosperm of *Borassus* (Fig. 14) represent an extreme case of the type of division just mentioned, wherein the daughter chromosomes are held together at the kinetochore region even after repeated replications. A few of the chromosomes in such cells, however, do not undergo this type of replication but remain normal. Yu (1964) has also made similar observation in Chinese hamster cells *in vitro* after 1500 r x-irradiation. As the capacity for recurring replication is limited to certain chromosomes, it may be assumed to be governed by a special mechanism of preferential replication.

The conditions such as 4x, 7x, 9x occasionally observed in the endosperm under the present study may result from preferential replication of certain sets of chromosomes. Cells showing replication of certain chromosomes with the rest unreplicated have been observed in this investigation. The same mechanism may also lead to aneuploidy which is often found in the endosperm of *Borassus*. Aneuploidy has been reported in the endosperm of a wide variety of plants (Straus 1954, Kapoor 1963, Abraham *et al.* 1965, 1966) and this has been attributed to multipolar mitoses and laggards. Multipolar mitoses were also observed in the endosperm under the present study.

The frequency of chromosome breakages (1.9%) observed in the endosperm of *Borassus flabellifer* is insignificant compared to the situation in the endosperm of many other plants investigated by the author (unpublished data). Biese (1958) has cited examples of chromosome breakage caused by c-mitotic agents. Accord-

ing to him such breakages are also possible at the kinetochore region. The minute diplochromosome like structures found in Fig. 10 may be the result of such a process. It is also possible that these represent the type of "dwarf chromosomes" found in many stem lines of tumours (Oberling and Bernhard 1961). Nichols, Levan, Aula and Norrby (1964) have reported similar structures as a result of pulverization in a syncytium in LU 106 tissue culture cells exposed to measles virus.

The observations made in this study show that the nucleolar number is not an index to the ploidy status. This is in conformity with earlier reports (Tandon and Kapoor 1961, Kapoor 1963) on the endosperm of other angiosperms. The lack of correlation between the ploidy and nucleolar number in *Borassus flabellifer* seems to be the result of nucleolar fusion, evidences for which have been revealed in this study. An interesting feature observed in the nucleoli in the mature endosperm of *Borassus* is the presence of a very prominent central vacuole occupying most of the volume, thus giving the appearance of "ring-shaped nucleoli" about which Busch and Smetana (1970) have reviewed. According to these authors the occurrence of these nucleoli in some blood cells, spermatocytes and oocytes seem to be inter-related with cell maturation and differentiation. In the endosperm under the present study these nucleoli were observed in mature cells which had lost the capacity for division and got accumulated with eragastic substances.

In the mature cellular endosperm and also in the centrifugate from the liquid endosperm of mature nuts, giant nuclei with pseudopods and branches have been observed. Similar observations have also been made by Oberling and Bernhard (1961) and Cameron (1964) in cancer cells. According to them this may increase the surface area of nuclei and thereby enhance nucleo-cytoplasmic interactions. Such nuclei have been reported in the endosperm of many plants (Kapoor 1963, Abraham *et al.* 1965, 1966, Varghese 1971). Some of these authors have cited this as a symptom of endosperm degeneration. However, in *Borassus* the endosperm grows even after the fourth month and almost fills the entire nut cavity. Finally it hardens and becomes stony. Hence the observed nuclear lobing may be a contrivance to establish and maintain nuclear control over the growing cytoplasm, when the nucleus has lost its capacity for division. Figures suggesting amitosis have also been observed in the aged endosperm of *Borassus*. Amitosis has been found to occur in the endosperm of several plant species (Abraham *et al.* 1965, 1966, Kapoor 1963). As these figures were observed in aged cells only, during this investigation, the chance for the daughter nuclei formed by amitosis to give rise to aneuploidy is very remote.

The frequent occurrence of endopolyploidy, aneuploidy, multipolar mitosis, nuclear polymorphism, etc. in the endosperm of *Borassus flabellifer* and unpublished observations on the endosperm of a number of other plants show similarity to the situation in cancer cells. Endopolyploidy has been reported in cancer cells by many authors (Levan and Hauschka 1953, Oberling and Bernhard 1961, Levan 1967, 1969). The occurrence of aneuploidy (Oberling and Bernhard 1961, Reisman, Zuelzer and Thompson 1964, Kato 1968, Levan 1969) and multipolar mitoses (Oberling and Bernhard 1961) have also been recorded in cancer cells. Nuclear polymorphism is a salient feature in advanced cancers (Oberling and Bernhard 1961,

Cameron 1964). The minute diplochromosome like structures observed in some cells of *Borassus* endosperm (Fig. 10) bear striking similarity to the "dwarf chromosomes" described by Oberling and Bernhard (1961) in many tumours. Almost all the above abnormalities suggesting similarity with chromosome behaviour in cancer cells have also been reported in the endosperm of the Philippine makapuno coconuts which also shows unlimited growth so as to fill the entire nut cavity at maturity (Abraham *et al.* 1965, 1966).

Summary

The cytology of the endosperm in palmyrah palm (*Borassus flabellifer* Linn.) was studied. During development of the endosperm, the free nuclear stage is followed by differentiation into peripheral cellular endosperm and the central liquid endosperm. At full maturity, the entire nut cavity is filled by the proliferating cellular endosperm. The endosperm was found to be predominantly triploid ($3x=54$). Cells showing multiples of triploid number of chromosomes such as $6x$, $12x$, $24x$, $48x$, $96x$ etc. also were seen in increasing frequency in the mature endosperm. This endopolyploidy was found to be the result of a type of division comparable to c-mitosis. Highly contracted "chromosome bundles" in triploid number were observed in a few cells. Euploid increase such as $4x$, $7x$, $9x$ etc., aneuploidy, multipolar mitosis, multiple bridges at anaphase, persistent bridges at telophase, nuclear polymorphism and amitosis were also observed. The nucleolar number was not in strict correlation with the ploidy status. "Ring-shaped nucleoli" were also constantly seen in mature endosperm.

The frequent occurrence of endopolyploidy, aneuploidy, multipolar mitosis, nuclear polymorphism etc. in the endosperm of *Borassus flabellifer* and unpublished observations on the endosperm of a number of other plants show similarity to the situation in cancer cells.

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