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Original article

Cytochemical and immunocytochemical study of nuclear structures of *Lacandonia schismatica*

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Summary – The interphase nucleus of the cells of several tissues of *Lacandonia schismatica* was studied using electron microscopy cytochemical and immunocytochemical methods. The EDTA staining procedure, preferential for RNP, contrasted the *Lacandonia* granules and perichromatin fibrils. These granules were found to be relatively resistant to RNAse hydrolysis, but they were easily digested if RNAse treatment was carried out after a short hydrolysis with pronase. Bismuth oxynitrate stained granular structures about 17 nm in diameter and the periphery of a few *Lacandonia* granules. The anti-snURNPs bound to RNP-containing fibrils in the perichromatin and interchromatin space and also to the periphery of some *Lacandonia* granules. Immunolabeling of DNA demonstrated numerous filaments of extended chromatin in the perichromatin and interchromatin spaces which were closely related to *Lacandonia* granules. These observations suggested that *Lacandonia* granules are equivalent to Balbiani ring granules of nuclei with polytene chromosomes and to perichromatin granules of other plant and animal nuclei. The small number of *Lacandonia* granules labeled in their periphery by anti-snURNP mAb were interpreted as being immature granules in the process of formation. The external or annular part of the ring-shaped structures is heavily labeled by anti-URNP mAbs but scarcely stained by the EDTA procedure. These features indicate that this region contains abundant proteins associated with snURNAs but probably little snURNAs. The synaptonemal-like complexes previously found in the interphase nuclei of *Lacandonia* are formed by two parallel masses of compact chromatin, which react with anti-DNA, and a central clear space crossed by fiber. Most of these fibers do not react with tests for DNA or RNA and are probably composed of proteins. The EDTA procedure contrasts some fibrils at the internal aspect of the masses of compact chromatin, suggesting the presence of remnants of the lateral element of a synaptonemal complex.

ribonucleoproteins / *Lacandonia* / plant nucleus / snURNPs / interphase

Introduction

Lacandonia schismatica E Martínez and CH Ramos [18] is characterized by the inverted position of the androecium in relation to the gynoecium [17, 18], a feature never seen before among other angiosperms [4, 5, 11, 25]. This remarkable feature and the restricted distribution of their population suggested that *L schismatica* originated through a macroevolution event. This possibility prompted a comparative ultrastructural study of the nucleus of *L schismatica* and other related plants [12]. The use of standard staining methods as well as the EDTA procedure preferential for RNP [2] allowed the finding of several peculiar features in the nuclei of *Lacandonia schismatica* and *Triuris brevystilis* [12]. Both plants belong to the division Magnoliophyta, class Liliopsida, subclass Alismatidae, order Triuridales. One of the main findings were the large clusters of a type of spherical granules, stained dark by Bernhard's EDTA procedure, 32 ± 3 nm in diameter, in the interchromatin and perichromatin space of nuclei of several tissues of these plants. As they were seen for the first time in cells of *L schismatica* and their size and spatial distribution are intermediate between interchromatin and perichromatin granules of vertebrate cells [21], they were termed '*Lacandonia* granules'. Interchromatin granules were not observed in nuclei of *L schismatica* or *T brevystilis* and perichromatin granules are scarce [12]. Other distinctive features of the nucleus of these plants are the ring-shaped structures and the synaptonemal-like complexes. The ring-shaped structures were found to be formed by an external ring of fibrillar material densely stained by standard uranyl-lead staining method and a central clear region positively contrasted by the EDTA procedure preferential for RNP. The synaptonemal-like complexes were described in many somatic cells as a narrow clear space of about 40 nm extending along elongated heterochromatin structures. These observations show that RNP particles and other nuclear structures of the cells of *L schismatica*, *T brevystilis*, and probably other Triuridales, differ from those of other plants suggesting that these species have a common ancestor with an evolutionary deviation from the major pattern of nuclear structure and cytophysiology [12].

The aim of this work is to characterize cytochemically and immunocytochemically the nuclear structures characteristic for *L schismatica* and other Triuridales in order to

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contribute to the understanding of their cytophysiological significance.

Materials and methods

Lacandonia schismatica E Martínez and CH Ramos were collected at the forest (Selva Lacandona, Chiapas, Mexico) and transported alive with their substrate to the laboratory where they were processed. Ovaries, tepals and complete flowers were fixed either in 6% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) or in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.16 M phosphate buffer (pH 7.2). Both fixations were carried out under vacuum for 2 to 3 min and subsequently for 1 h at room pressure and temperature. After several rinses in buffer, the samples were dehydrated in graded series of ethyl alcohol starting 30% and embedded in LR White or in an epoxy resin (glycide ether 100, Merck). The dehydration and infiltration with LR White were carried out at 4°C. Bernhard's EDTA procedure preferential for RNP [2] was used for morphological study of *Lacandonia* granules and other RNP containing structures. Bismuth oxynitrate staining was performed before embedding as well as on sections according to Locke and Huie [15]. Samples or sections of rat liver were stained at the same time, using the same solutions, as positive control.

A commercial monoclonal IgM recognizing DNA (Progen) and an anti-Sm monoclonal IgG (Y12) that recognizes a phylogenetically conserved epitope common to RNP particles containing U1, U2, U5 and U4/U6 snRNA [10, 14, 16], were used in our experiments. Indirect immunolabeling was performed on ultrathin sections using a secondary antibody complexed with colloidal gold according to Biggiogera *et al* [3].

Enzymatic hydrolysis was performed on thin LR White sections handled with Marinozzi rings. The concentrations of enzymes and times of treatments were as follows: RNAse 2% in buffer Tris-HCl 10 mM (pH 7.4), 2 to 7 h; pronase 0.3% in the same buffer, 4 h. Double digestions were carried out on the same section: pronase 0.3%, 15 min followed by RNAse 2%, 7 h. All digestions were performed at 37 °C. Controls were floated on the buffer for the same time.

Morphometry

The size of the granules stained with bismuth oxynitrate and EDTA procedures was estimated by measuring their larger diameter in high magnification pictures. Darkly contrasted granules were systematically chosen in 25 pictures.

Results

Figure 1a shows the position of one nucleus in a flower bud of *L schismatica*. The reticulate distribution of compact chromatin of the nuclei of the internal and external teguments of the ovary can be observed in light micrographs (fig 1b, c).

RNP containing structures

Electron micrographs of sections of the teguments of the ovary stained with the EDTA procedure for RNP show large clumps of *Lacandonia* granules, darkly contrasted, occupying the interchromatin and perichromatin spaces (fig 2a). These granules occur also isolated or in small groups in cells of different tissues. Frequently a small group of *Lacandonia* granules appear at the tip of a filament, which branches from a central filament, resembling a transcription unit [20] (fig 2b). This EDTA staining procedure also contrasts some fibrillar structures located in the internal edge of the heterochromatin strands of synaptonemal-like complexes, darker than other chromatin fibrils. Few or no dark fibrils keep their contrast in the central clear space (fig 2c). As previously described [12], the EDTA staining procedure contrasts the internal portion of the ring-shaped structures 0.5 to 0.9 μm in diameter, frequently found in the nucleoplasm of *Lacandonia* tegumentary cells. The external region of these annular structures is stained by uranyl acetate and then bleached by EDTA. However, a few fibrils keep the contrast in the external ring. These fibrils are frequently in continuity with EDTA positive filaments in the nucleoplasm.

The comparison of control sections (fig 3a) and those floated on RNAse (fig 3b) indicates that *Lacandonia* granules are relatively resistant to the RNAse hydrolysis for 2 to 7 h. However, when a short treatment with pronase is applied before the RNAse digestion, most of *Lacandonia* granules disappear. The remaining granules are smaller than the untreated ones and frequently appear deformed (fig 3c). However, scattered granules about 17 nm in diameter are resistant to RNAse extraction and even to the mentioned double hydrolysis (fig 3c).

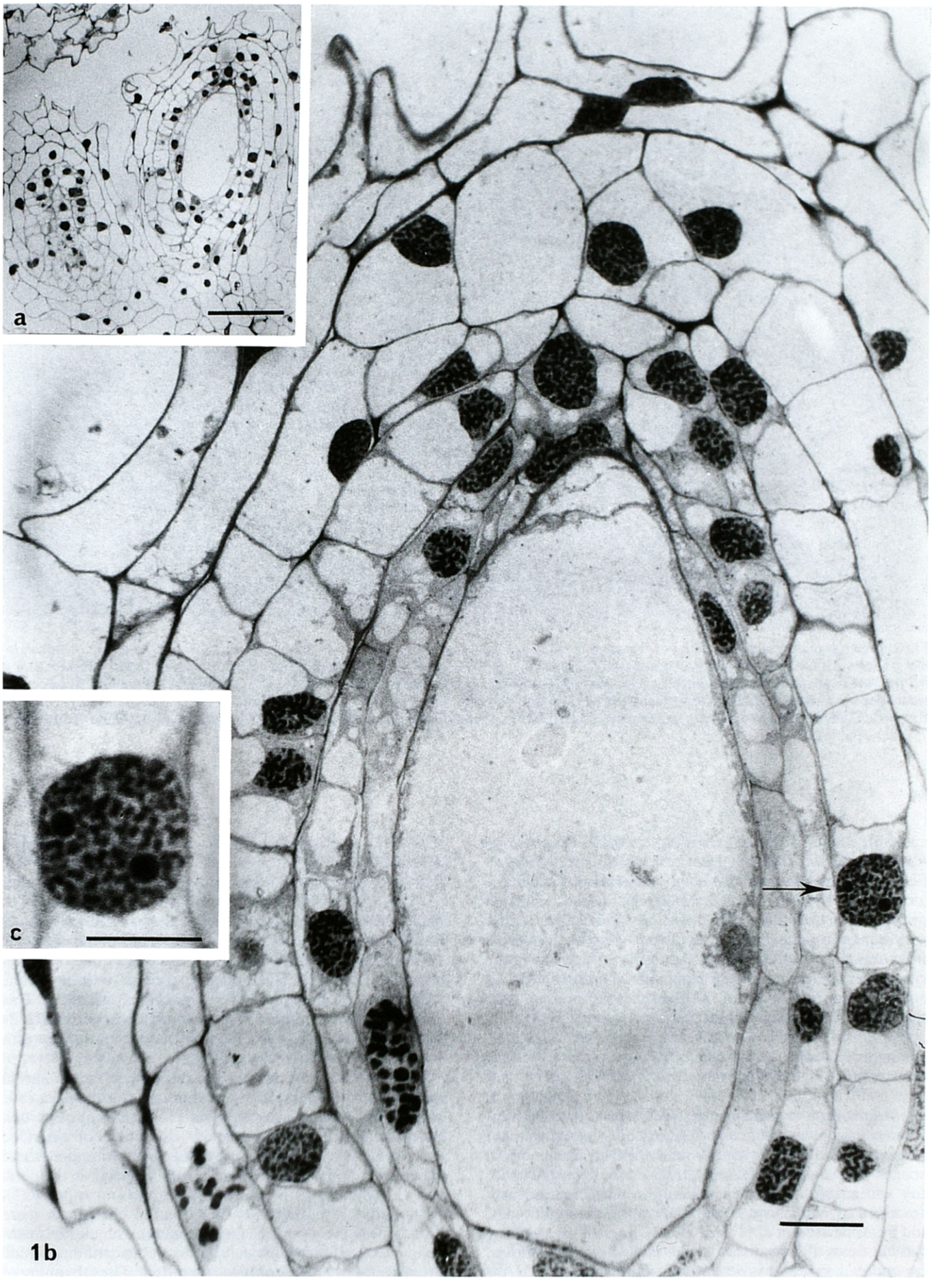
Bismuth oxynitrate staining was performed either post- or pre-embedding, in order to characterize further the small granular structures resistant to RNA extraction. It contrasts some perichromatin fibrils and granular structures much smaller than *Lacandonia* granules (fig 4). Several nuclear structures such as nucleolus and chromatin stain clear gray when bismuth oxynitrate staining is performed after 6% glutaraldehyde fixation. The gray level of chromatin is used as the standard negative level of staining. *Lacandonia* granules are never darker than chromatin. However, the periphery of a few granules is sometimes darkly stained (fig 4). Measurements of the small granules stained with bismuth indicate that they are 17 ± 2.5 nm (SD) in diameter.

The anti-snURNP antibody binds to perichromatin fibrils and to RNP fibrils in the interchromatin space and between *Lacandonia* granules. However, *Lacandonia* granules were not decorated by this antibody (fig 5). A few of these granules display immunolabeling associated with their periphery. It is interesting to note that Y12 labels densely the external dense region of the ring-shaped structures (fig 5, inset).

Chromatin

The immunolabeling of DNA demonstrates that labeled fibrils of extended chromatin intermingle with *Lacandonia* granules in the interchromatin space (fig 6a). Inside the

Fig 1. Light micrographs of a longitudinal section of a flower bud of *Lacandonia schismatica*. Toluidine blue staining. **a.** Low magnification in which two carpels can be observed. 143 ×. Bar = 100 μm. **b.** Larger magnification of the ovule shown in the previous picture. The external and the internal teguments can be observed. Nuclei display a reticular arrangement of the chromatin. The arrow points to a nucleus with two compact nucleoli. 1620 ×. Bar = 10 μm. **c.** A high magnification view of the nucleus pointed out. The network of compact chromatin is clearly visible. 2213 ×. Bar = 10 μm.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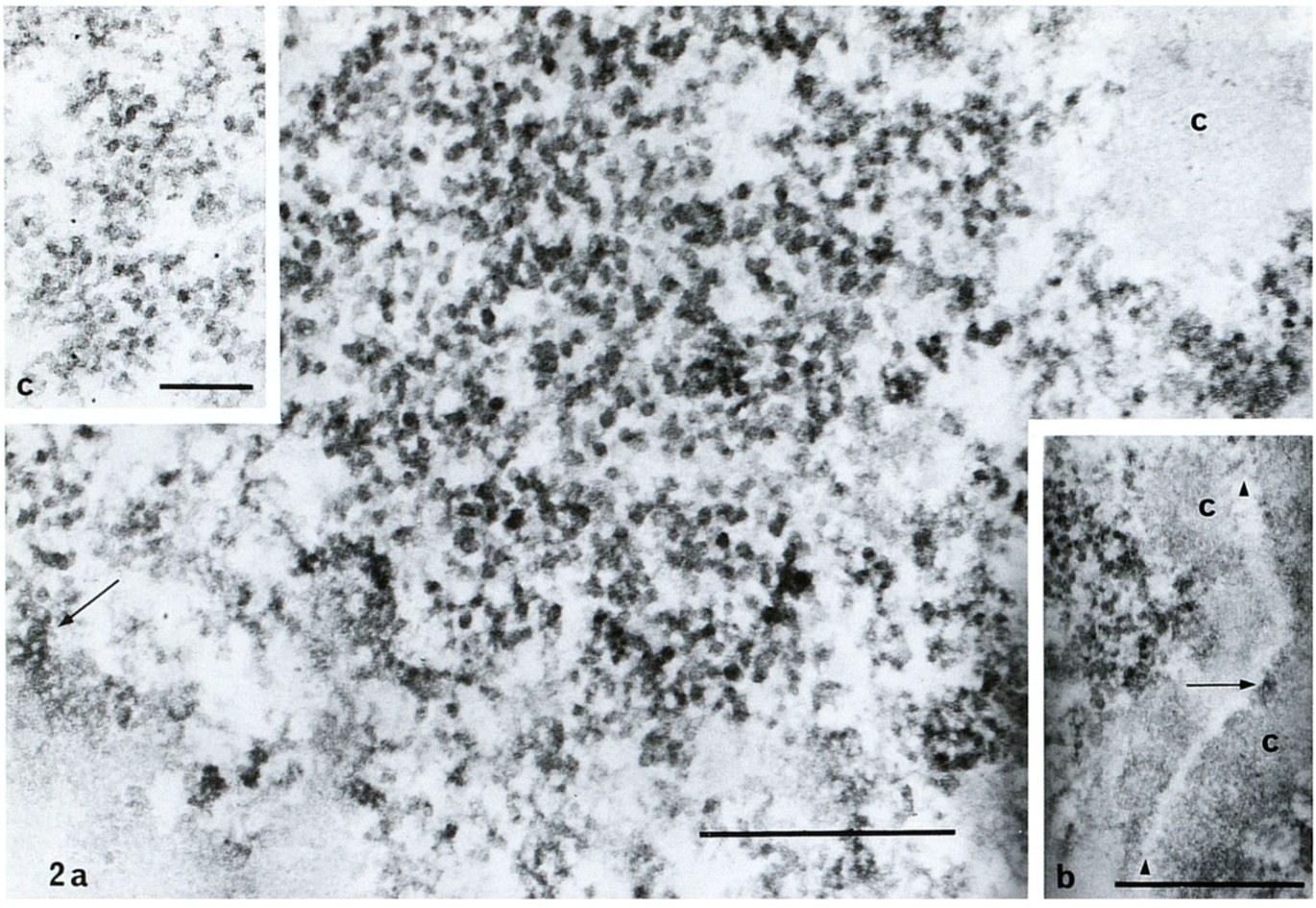

Fig 2. Electron micrographs of nuclei of tegumental cells. **a.** EDTA staining procedure preferential for RNP. A large group of *Lacandonia* granules darkly stained. Compact chromatin (c) is clear gray. The arrow points to dark stained perichromatin fibrils. 72500 ×. Bar = 0.5 μm. **b.** A synaptonemal-like complex stained by the same procedure. The two strands of compact chromatin (c) are gray. The central clear space is crossed by a few fibrils (arrow head). The internal edge of one of the chromatin strands is dark stained (arrow). 52000 ×. Bar = 0.5 μm. **c.** Branched fibrogranular cluster resembling a spread transcription unit stained with uranyl acetate and lead citrate. 75000 ×. Bar = 0.25 μm.

nucleolus there is a strong labeling on the heterogeneous fibrillar centers [24] and a weak decoration of the dense fibrillar component and the homogenous fibrillar centers (not shown). The ring-shaped structures are seldom labeled by anti-DNA mAb, however, fibers of extended chromatin are frequently seen in contact with the periphery of these structures (fig 6, inset). The two dense lateral regions of the synaptonemal-like complexes react strongly with anti-DNA mAb, but the fine fibrils of the clear central zone are only sparsely decorated (fig 6, inset).

Discussion

Our results demonstrate several structural and cytochemical similarities between *Lacandonia* granules, perichromatin granules and Balbiani ring granules. In fact, the branched images (Christmas trees) seen in the present study without any spreading, closely resemble the transcription unit described by Miller and Beatty [20] in different cell types, and by Andersson *et al* [1] in Balbiani rings of the polytene chromosomes of the salivary glands of *Chironomus tentans*. *Lacandonia* granules are relatively insensitive to RNAse but most of them disappear or become smaller and deformed when a short pronase treatment is applied prior to RNAse extraction. Perichromatin granules [21] and Balbiani ring granules [27] react similarly toward these treatments. Bismuth staining carried out after glutaraldehyde fixation does not contrast the *Lacandonia* granules or the perichromatin granules in the controls performed on mammalian liver cells. However, a discontinuous staining can be seen in the periphery of some *Lacandonia* granules. This staining may be related with the presence of snURNPs detected by immunocytochemistry in the periphery of a small number of *Lacandonia* granules.

The labeling of fine fibrils in the perichromatin and in the interchromatin space corroborates the observations made with phosphotungstic acid [11], and suggests that *Lacandonia* granules are related with fibrils of extended chromatin. The relations between *Lacandonia* granules and extended chromatin are similar to those existing in the puffs and Balbiani rings of the polytene chromosomes [27]. Although a few filaments of extended chromatin were found inside the groups of interchromatin granules in mammalian liver cells [6], they are far less abundant than inside the clumps of *Lacandonia* granules. The absence of

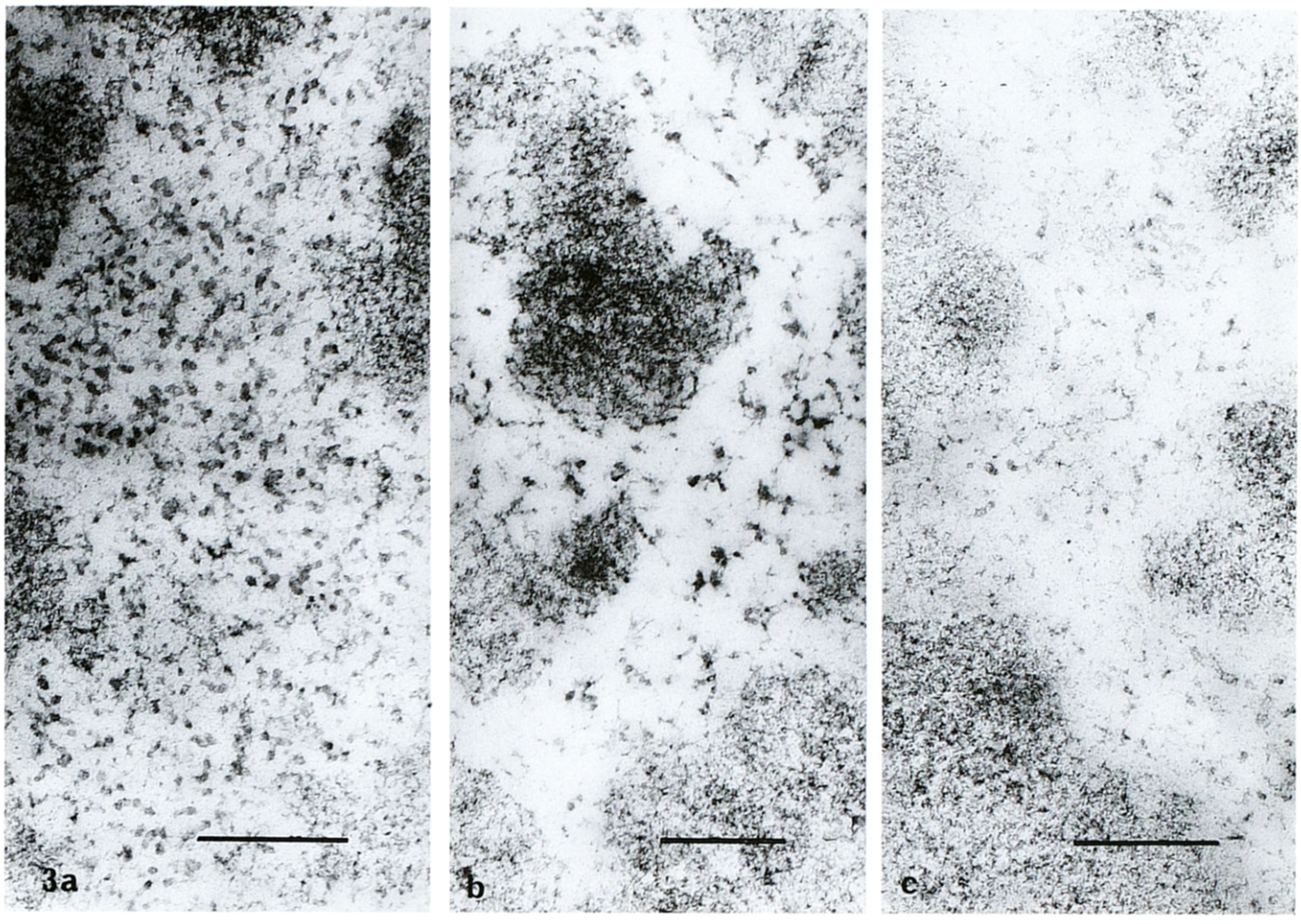

Fig 3. Nuclei of tegumental cells. Uranyl acetate and lead citrate staining. Bars = 0.5 μm. **a.** Control floated on 10 mM Tris-HCl (pH 7.4) for 2 h. The *Lacandonia* granules are abundant and they show normal morphology and size. 41 500 ×. **b.** Section floated on 2% RNAse in the same buffer for 2 h. *Lacandonia* granules are fewer and appear distorted. 35 000 ×. **c.** Double digestion with 0.3% pronase in Tris-HCl buffer for 15 min and then with 2% RNAse for 2 h. Only a few remnants of *Lacandonia* granules can be seen. 40 000 ×.

snURNPs in most of *Lacandonia* granules as well as its presence in the periphery of a few of them correspond with the distribution of these proteins found in Balbiani ring granules of the nuclei with polytene chromosomes [29]. SnURNPs are present only in immature Balbiani ring granules which are forming in regions active in transcription, but they are not found in the nucleoplasmic granules [29]. Thus, labeled *Lacandonia* granules probably correspond also to structures in the process of formation. Pioneer ultrastructural immunocytochemical localizations of snRNPs in mammalian nuclei demonstrated that they associate mainly to perichromatin fibrils, interchromatin granules and coiled bodies and occasionally to perichromatin granules [9, 23]. Recent studies using high resolution *in situ* hybridization demonstrate that the snRNAs have a similar distribution [31]. All this evidence supports the view that *Lacandonia* granules correspond to perichromatin of other plant and animal cells [13] and Balbiani ring granules of salivary gland of the larvae of Diptera [27].

In samples fixed in cold aldehydes, dehydrated and embedded at low temperatures, granules 30 to 50 nm in diameter were described in the interchromatin space of different tissues of angiosperms. These structures are reported to stain positively with Bernhard's EDTA procedure preferential for RNP and not to react with anti-snRNP antibodies [26]. *Lacandonia* granules differ from these structures in several aspects. The granules found in other angiosperms are polymorphic and highy variable in diameter [26], while *Lacandonia* ones are spherical and their diameters are grouped in narrow intervals (30 to 35 nm) [12], resembling in these features to the perichromatin granules described in plant non-reticulate nuclei [7].

Interchromatin granules were described in different plants [13] and their preferential staining with bismuth oxynitrate corroborated in reticulate nuclei (*Allium cepa*, monocotyledon) [19] and in non-reticulate nuclei (*Lycopersicum esculentum*, dicotyledon) [22]. This staining method contrasts granules about 17 nm in diameter scattered in the nucleoplasm of the nuclei of *L schismatica*. Although the size of these granules is smaller than those of other plants, their dispersed distribution and their resistance to RNAse and pronase-RNAse hydrolysis strongly suggest that this bismuth positive granules correspond to interchromatin granules.

The abundant binding of the anti-snRNP mAb Y12 to the external dense region of the ring-shaped structures

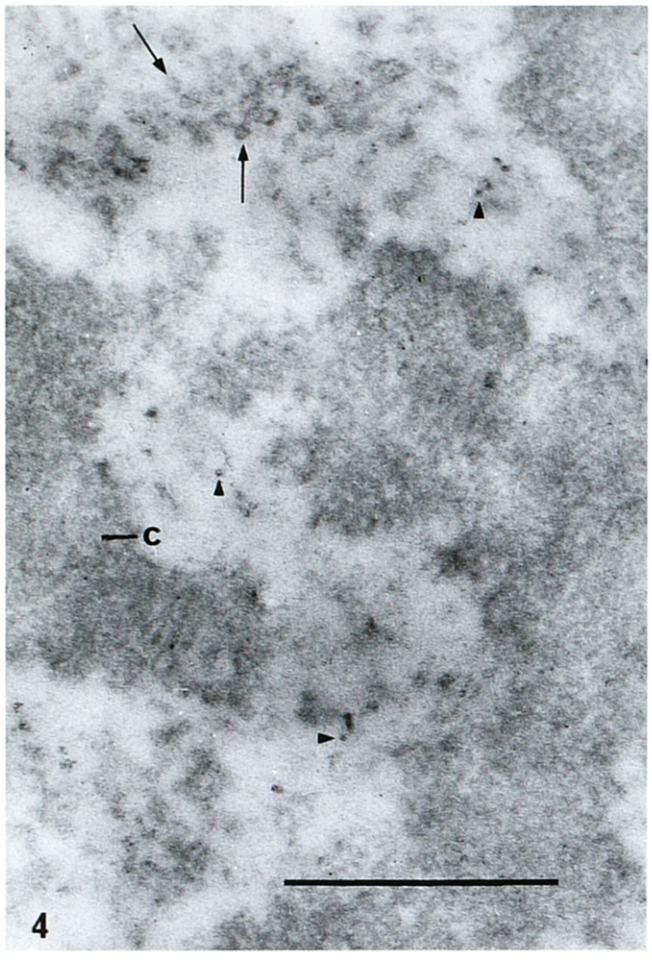

Fig 4. Bismuth oxynitrate staining. The gray shade of the compact chromatin (c) is the negative level of the reaction. Small granules depict an intense staining (arrow heads), some *Lacandonia* granules are stained at the periphery (arrow), most of them are not darker than chromatin. 70000 ×. Bar = 0.5 μm.

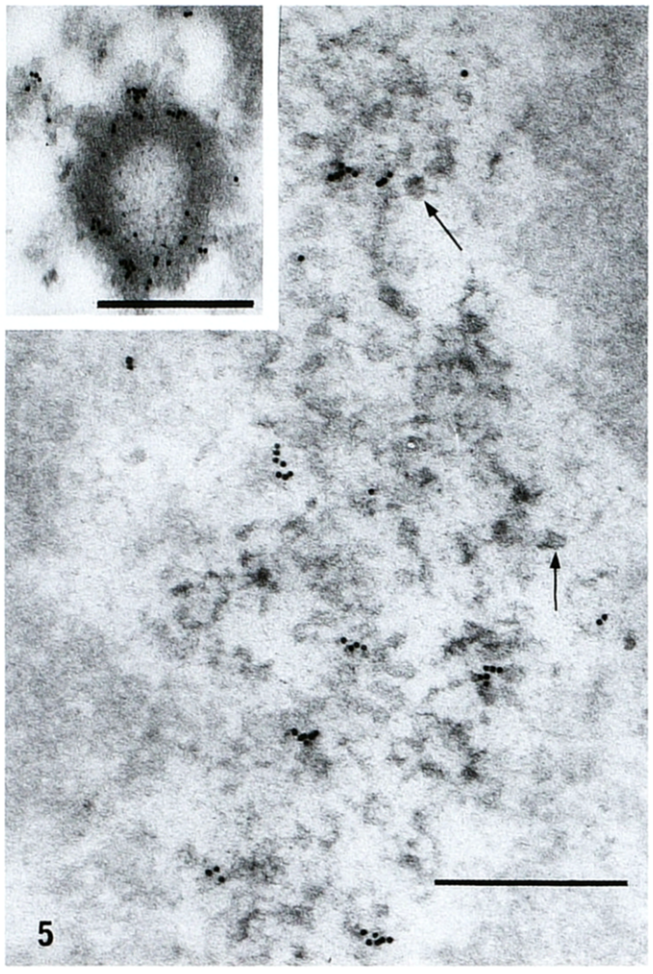

Fig 5. Distribution of anti-Sm monoclonal antibody. Most of the labeling is associated to fibrils. *Lacandonia* granules (arrows) are not decorated. 100000 ×. Bar = 0.25 μm. The inset shows an intense labeling of the external annular component of a ring-shaped structure. 80000 ×. Bar = 0.25 μm.

which is scarcely stained by EDTA procedure preferential for RNP [12], suggests that this region contains the higher concentration of proteins associated with snURNAs but little RNA. It is worth noting that the filaments stained by EDTA procedure in the external annular part are frequently in connection with nucleoplasmic-RNP containing fibrils. On the contrary, the central part of the ring-shaped structures is positive stained by EDTA procedure [12], but is scarcely labeled by Y12, suggesting that this RNA does not correspond necessarily to snURNA. It is of interest to note that the ring-shaped bodies are surrounded by filaments of extended chromatin associated to RNP-containing structures, frequently labeled by Y12. At the present moment the cytophysiological role of these structures is not understood.

Immunolabeling with anti-DNA mAb corroborates that the synaptonemal-like complexes correspond to a parallel arrangement of clumps of compact chromatin. However, only very few of the fine fibrils bridging the central clear space appear labeled by this antibody. The EDTA-positive fibrils of the inner part of the heterochromatin strands are similar to the remains of the lateral elements of meiotic synaptonemal complexes in diplotene stage. All these features of the synaptonemal-like complexes of *L schismatica* are similar to synaptonemal complexes found in meiocytes in late pachytene or early diplotene stages of meiotic prophase [7, 28, 30]. Similar structures were found by Esponda in microspores of *Allium cepa*, a post-meiotic cell, and were interpreted as relicts of synaptonemal complexes [8]. As first meiotic division separates homologous chromosomes, the presence of synaptonemal-like complexes binding heterochromatic strands in the interphase nuclei of various somatic cells cannot be explained as remnants of meiotic structures. Thus, these complexes must form after reductional division. They may be the structural support of some kind of somatic pairing carried out by a mechanism resembling meiotic pairing of homologous chromosomes taking place in this group of plants (Triuridales).

Acknowledgments

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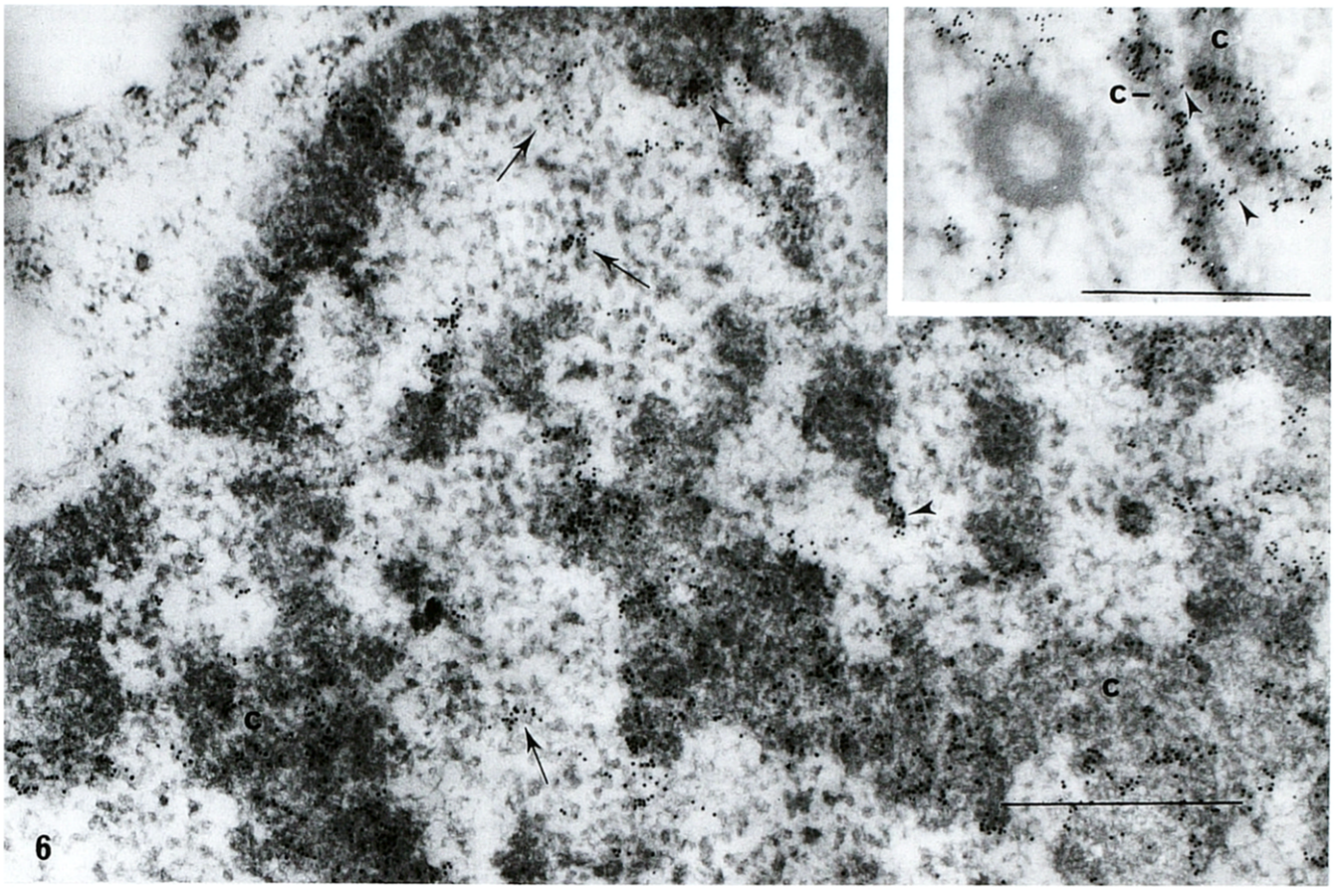

Fig 6. Distribution of anti-DNA monoclonal antibody. Compact chromatin (c) is heaveily labeled. Gold grains are also found in the perichromatin region (arrow head) and intermingled with *Lacandonia* granules in the interchromatin space (arrows). 64000 ×. Bar = 0.5 μm. The inset shows intense labeling of the compact chromatin (c) strands of a synaptonemal-like complex and a few gold grains are localized in the clear central space (arrow head). The ring-shaped structure is not labeled but some of the fine filament irradiating from it are densely decorated. 60000 ×. Bar = 0.5 μm.

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