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Histochemical and ultrastructural analysis of latex vessels in the dormant embryo of *Euphorbia marginata*

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Abstract

Cytological and histochemical aspects of the embryonal laticifers of *Euphorbia marginata* were studied by conventional, fluorescence and electron microscopy. Results indicate that laticifers are distinguishable from adjacent cells due to the small dimensions of cytoplasmic inclusions and the high content in proteins of the cytoplasmic matrix. Differences between outermost and innermost laticifers were observed in respect to their morphology and contents. Innermost laticifers contain glycoproteins and sulphated proteins, they have nuclei with «compact» nucleoli and a thin cell wall. Outermost laticifers show a thick cell wall, a cytoplasm reacting strongly positive with fluorescent PAS and nuclei with «loosely structured» nucleoli. These histochemical and cytological differences together with previously published ontogenetical data suggest a different metabolic role for inner and outer laticifers in the embryo.

Introduction

Non-articulated laticifers start their growth in the plant immediately after their initiation in late embryogenesis to form a complex embryonal latex system (Mahlberg, 1960, 1961; Mahlberg and Sabharwal, 1968). During germination, the laticifers change in distribution, cytological morphology and chemical composition (Mahlberg, 1959; Moor, 1959; Moritz and Frohne, 1967). Although several indications regarding the latex composition are available (Tunnicliffe, 1954; Bateman, 1963; Groeneveld, 1976), the development and the morphology of the laticiferous system are incompletely understood. We think that cytological and histochemical studies of embryonal laticifers can shed some light on the evaluation of the functional role of these enigmatic cells from late embryogenesis to seedling formation.

In the embryo of *Euphorbia marginata,* laticifers grow with a precise pattern under endogenous and exogenous stimuli that determine the formation of deep laticifers and successively of superficial laticifers (Bruni *et al.,* 1978; Bruni, 1979). This paper proposes to check if the previously observed ontogenetical differences between deep and superficial laticifers in the embryo of *E. marginata* also reflect diversities in the chemical content and ultrastructural organization of laticifers.

Materials and methods

Quiescent embryos removed from seeds of *E. marginata* Pusch were immediately processed to make both frozen and plastic-embedded sections. For preparation of frozen sections, embryos were immersed in 5 % aqueous polyvinyl alcohol, and frozen at −35 °C (Chayen *et al.*, 1960). A cryomicrotome (Kryomat Leitz), with methanol as freezing fluid at −40 °C, was used for cutting both longitudinal and cross sections (20-30 μm). Frozen sections were cut from fresh unfixed embryos, or from embryos fixed in aldehyde fixatives.

A) Fixation and embedding _ Embryos were fixed as follows: *a)* buffered formalin (4 g paraformaldehyde dissolved in 100 ml 0.2 M phosphate buffer, pH 7.0) for 5 h at 4 °C; *b)* formalin -mercuric chloride-acetic acid mixture, for 12 h by the method of Dall'Olio *et al.* (1978). After fixation the material was washed, dehydrated with an ethanol series or with ethylene glycol monomethyl ether and embedded in butyl-methyl methacrylate containing 0.8 % a,*a*-azobis (isobutyronitrile) as catalyst (Feder and O'Brien, 1968). One part of the embryos was embedded in glycol methacrylate by Ruddell's method (1967). This medium allows the preservation of lipids and other compounds which are otherwise extracted by conventional embedding procedures. The specimens were then cut with an LKB-Pyramitome at 1−3 μm, using dry glass knives.

B) Cytochemical techniques _ Frozen or plastic-embedded sections were processed by the following histochemical methods: *a)* Sudan black B (Jensen, 1962; Bronner, 1975), osmium tetroxide (Jensen, 1962), peracetic acid-Schiff (PAAS) (Jensen, 1962) for lipids; *b)* $SbCl_3$ (Mace *et al.*, 1976) for terpenoid aldehydes; *c)* Hoepfner-Vorsatz test (Reeve, 1951), primary fluorescence (Ibrahim and Towers, 1960), $FeCl_3$ reaction (Jensen, 1962), and anilin-KIO_3 (Mace, 1963) for polyphenols and quinones; *d)* Feulgen (Jensen, 1962), acridine orange (Busch and Smetana, 1970), azure B (Jensen, 1962), for DNA and RNA; *e)* ninhydrin-Schiff (Jensen, 1962), Hg-fluorescamine (Bruni *et al.*, 1976) for total proteins; *f)* silver impregnation (Stockert *et al.*, 1969) for nucleolar structure and basic proteins; *g)* organomercurials (Bruni and Fasulo, 1979) for sulfur-containing proteins; *h)* acriflavine (Bruni, 1979) for glycoproteins; *i)* periodic acid-Schiff (PAS) (Jensen, 1962), fluorescent PAS reaction (F-PAS) (Bruni and Vannini, 1973), potassium iodide (Johansen, 1940), IKI-H_2SO_4 (Jensen, 1962) for carbohydrates.

C) Control reactions – For all the above cited histochemical methods, control reactions were made following the suggestions of the respective author. In addition, sections were subjected to the control procedures proposed by Swift and Saxton (1967) in order to evaluate the PAAS, conventional PAS and F-PAS reactions. In particular, prior to treatment with Schiff's reagent, the sections were subjected to the following control techniques: *a)* block of aldehyde groups by dimedone, prior to peracetic or periodate oxidation; *b)* treatment again with dimedone after peracetic or periodate oxidation; *c)* treatment with a solution of bromine water (Ling-Lee *et al.*, 1977); *d)* pretreatment with acetic anhydride after dimedone blockage to provide further proof of the presence of 1,2-glycol groups (McManus and Cason, 1950).

D) Instrumentation and procedures – Preparations were observed and photographed with a Zeiss Photomicroscope II. For fluorescence microscopy, the optical system was equipped with an epi-fluorescence condenser (III RS), a Xenon lamp and suitable filter sets as recommended for the fluorochromes employed. For electron microscopy, the plant material was fixed in 3 % buffered glutaraldehyde and post-fixed in 1 % osmium tetroxide. The embryos were dehydrated in an alcohol series and embedded in Durcupan A/M-Epon 812 mixture. Sections of the material were obtained with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, viewed and photographed with a Jeol JEM-T7 instrument.

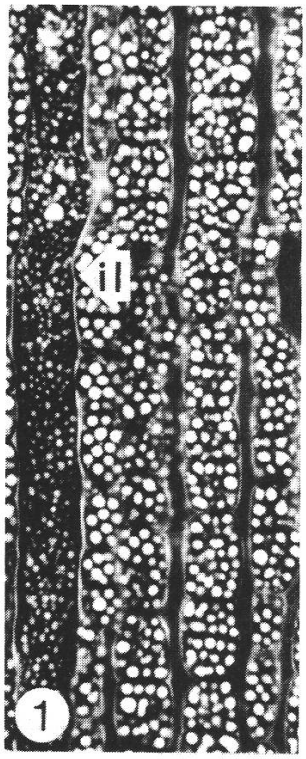
Results

By light and electron microscopy, embryonic laticifers of *E. marginata* appear filled with cytoplasmic inclusions smaller in size ($^1/_4$–$^1/_8$) than those present in the adjacent cells (Figs. 1, 14). In freshly frozen sections processed with classical methods for fats (Sudan black B, osmium tetroxide) and proteins (ninhydrin-Schiff, Hg-fluorescamine), these inclusions appear as closely packed vesicles of varying sizes. Using electron microscopy, it is possible to recognize several inclusions that are described according to the terminology proposed by Matile (1975) and Narthey (1978): *a)* wide vesicles showing, at high resolution, a single-opaque contour (Figs. 16, 17, 18), that are probably spherosomes because of their content positive in respect to lysochrome, primary fluorescence (Fig. 2) and PAAS tests; *b)* typical protein bodies or aleurone grains (Figs. 17, 18), which are strongly fluorescent by the Hg-fluorescamine method (Fig. 7); *c)* small vesicles

Table I. Histochemical comparison of substances detected in innermost and outermost laticifers

Substances	Histochemical tests	Innermost laticifers	Outermost laticifers
lipids	autofluorescence (!)	+	+
	Sudan Black B (!)	+ + +	+ + +
	Bronner's method	+ + +	+ + +
	osmium tetroxide	+ +	+ +
	PAAS	+ +	+
terpenoids	$SbCl_3$ (!)	−	−
phenols	$FeCl_3$	−	−
	Hoepfner-Vorsatz test (!)	−	−
	autofluorescence	−	−
quinones	anilin-KIO_3 (!)	−	−
DNA	Feulgen	+	+
	acridine orange	+ +	+ +
RNA	azure B	+	+
	acridine orange	+ + +	+ + +
total proteins	ninhydrin-Schiff test	+	+
	Hg-fluorescamine	+ + +	+ + +
basic proteins	silver impregnation	+ + +	+ + +
sulphated proteins	organomercurials	+ + +	−
glycoproteins	histofluorescent method	+ + +	−
carbohydrates (cytoplasm matrix)	PAS	+	+
	IKI (!)	−	−
	fluorescent PAS (F-PAS)	+ + (orange)	+ + (green)
carbohydrates (cell wall)	PAS	+	+
	IKI-H_2SO_4	+	+
	fluorescent PAS (F-PAS)	+ + (green)	+ + (orange)

(!) Freshly frozen sections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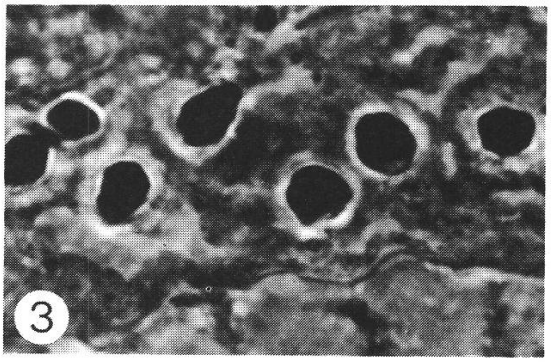
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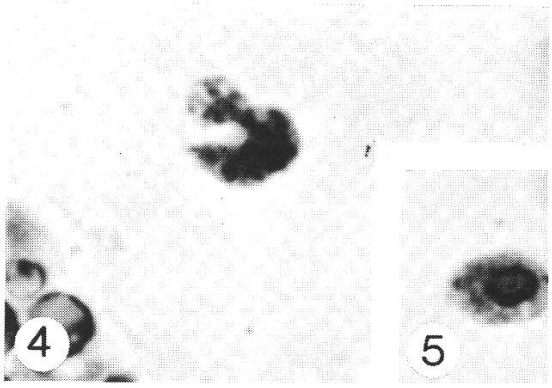
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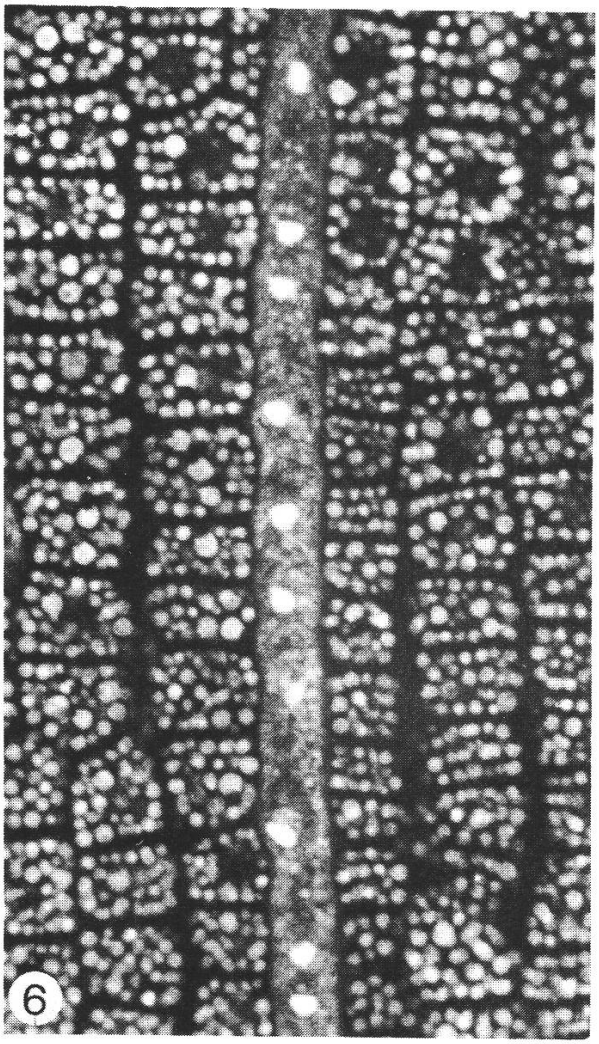
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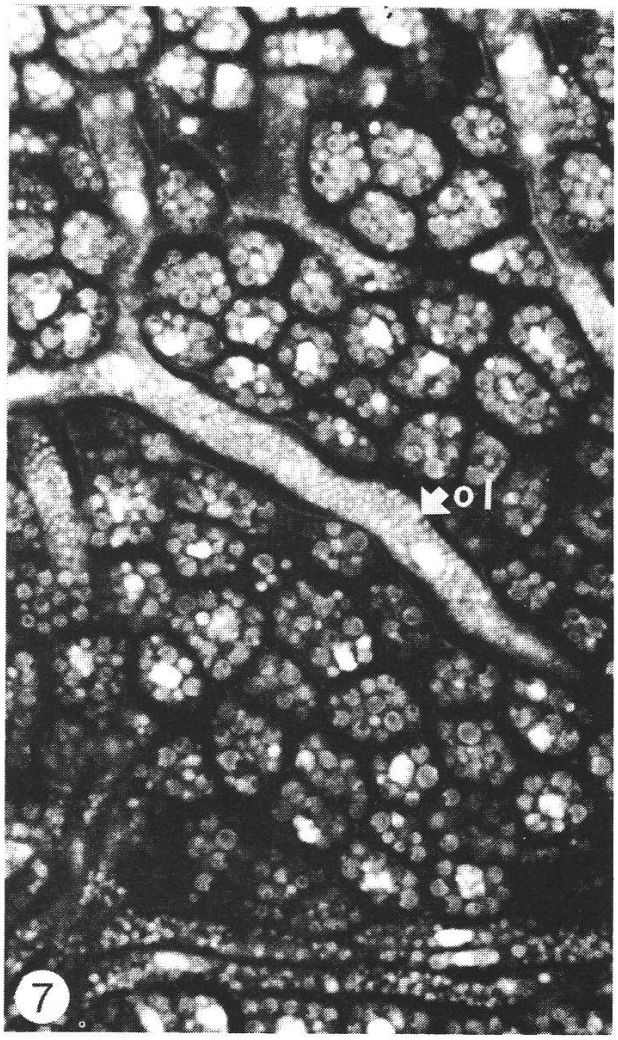
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having a double membrane contour which are referred to as prospherosomes when a thickening of the middle layer and a homogeneous content are present or as small inclusions when their contour, similar in thickness to that of ER-vesicles, envelops a heterogeneous content (Figs. 17, 18). The distribution of these inclusions in embryonic laticifers is not uniform. In innermost laticifers, protein bodies are more abundant and lipid vesicles larger than in outermost laticifers which, instead, have a high number of small inclusions and prospherosomes. The $FeCl_3$, Hoepfner-Vorsatz test and autofluorescence did not indicate the presence of hydrolizable and condensed tannins and polyterpenoids either in laticifers or in adjacent cells (Table I).

Nuclear chromaticity and morphology was studied by conventional and fluorescence methods. With silver impregnation, the nuclei appear stained ochreous yellow and the nucleolus dark brown while the surrounding cytoplasm is yellow in colour. By this conventional technique and by the fluorescent acridine orange method it is possible to observe a clear difference in nuclear morphology between inner and outer laticifers. In fact, in outermost laticifers, nuclei are irregularly distributed, have a largely lobed shape (Figs. 4, 5), and contain one or two «vacuolated» nucleoli which emit a strong orange fluorescence with acridine orange. In innermost laticifers, nuclei are ovoid, very numerous and regularly distributed. From the nodal plexus to the root apex, more than 45 nuclei can be observed in each vascular laticifer. In these nuclei which display a green fluorescence with acridine orange the nucleolus is often absent. When visible, the nucleolus appears of the «solid» type and emits a pale, green-yellow fluorescence with acridine orange. Other differences between outer and inner laticifers were observed by fluorescence methods for sulfur-containing proteins and glycoproteins. Fluorescent mercurials

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Explanation of the plates

Abreviations used in figures:

CRYO	=	frozen section	ol	=	outermost laticifer
cw	=	cell wall	pb	=	protein body
il	=	innermost laticifer	ps	=	prospherosome
n	=	nucleus	sp	=	spherosome

Fig. 1. Longitudinal section of hypocotylary axis with a laticifer containing small osmiophilic lipid and protein inclusions. Bronner's method. × 400.

Fig. 2. Longitudinal section of cotyledon (CRYO). Inclusions containing neutral fats are strongly autofluosrescent. × 600.

Fig. 3. Several nuclei with a compact structure and lacking nucleoli in an innermost laticifer. Silver impregnation. × 900.

Fig. 4. Nucleus with an amoeboid shape in an outermost laticifer. Silver impregnation. × 1,200.

Fig. 5. Nucleus with a «vacuolated» nucleolus in an outermost laticifer. Silver impregnation. × 1,000.

Fig. 6. Regularly shaped nuclei of an innermost laticifer. The nucleoli are not distinguishable. Acridine orange. × 500.

Fig. 7. Longitudinal section of cotyledon showing protein content in outermost laticifers. Hg-fluorescamine. × 500.

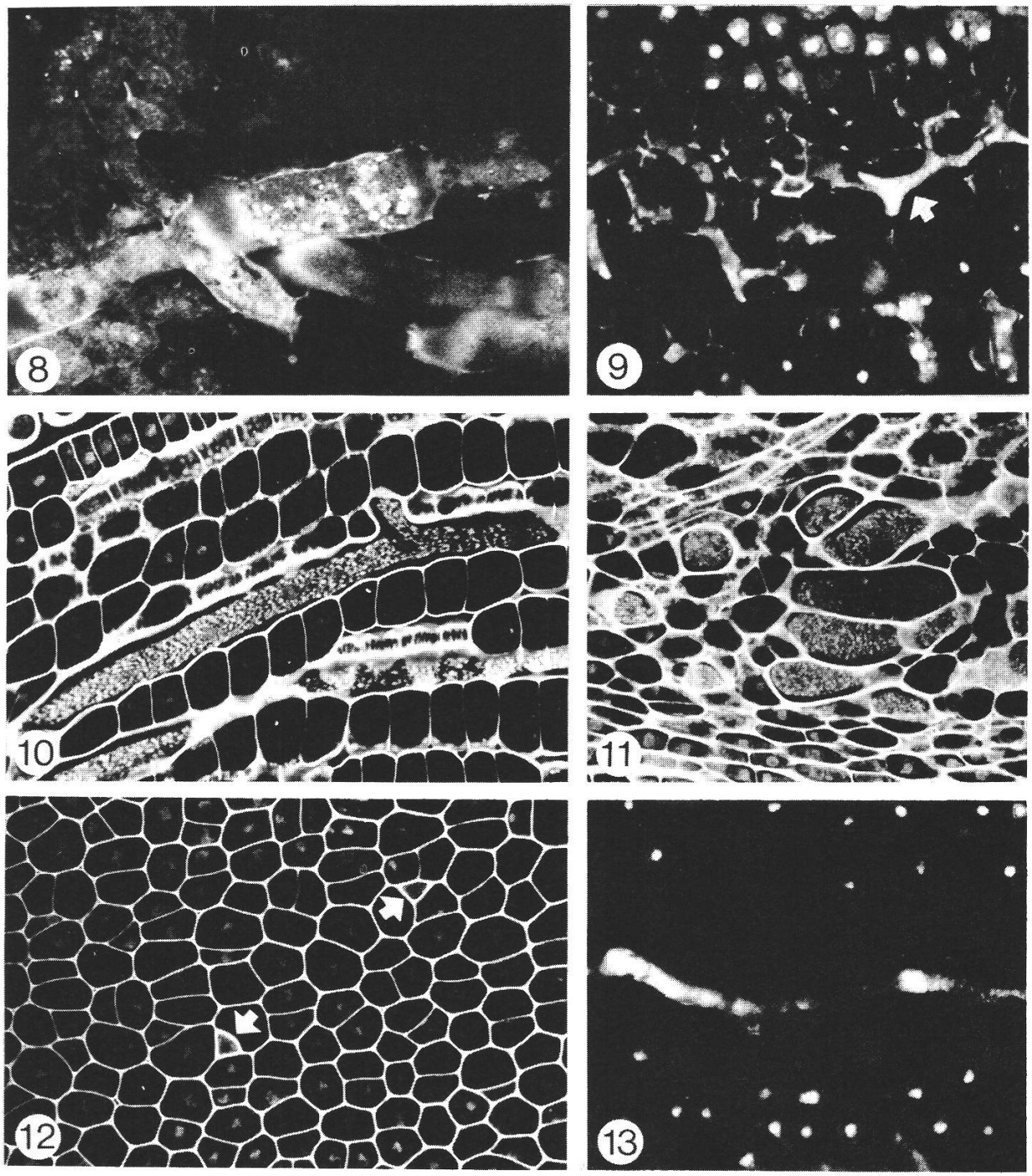


Fig. 8. Transverse section of latex plexus. The strong fluorescence of the cytoplasm in innermost laticifers indicates the presence of proteins containing sulfur. Organomercurial. × 600.

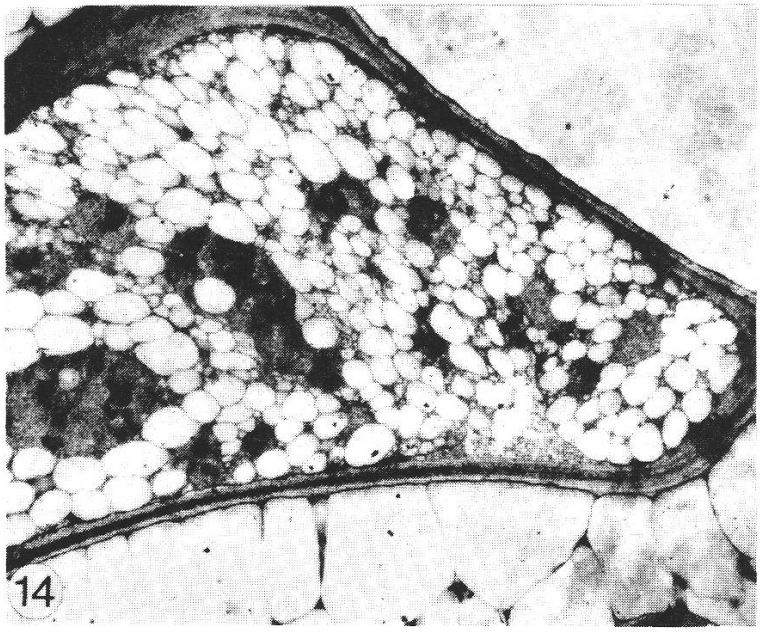
Fig. 9. Longitudinal section of cotyledon with outermost laticifers. Fluorescence of acridine orange shows an elongate nucleus (arrow). × 250.

Fig. 10. Longitudinal section of an innermost laticifer in hypocotyledon with content of PAS positive material. F-PAS. × 350.

Fig. 11. Ultraviolet micrograph showing transverse section of latex plexus and the fine granulation of PAS-positive material in the laticifer matrix. F-PAS. × 350.

Fig. 12. Transverse section of cotyledon. Cell walls of outermost laticifers (arrows) are thicker than those of the adjacent cells. F-PAS. × 350.

Fig. 13. Longitudinal section of inner laticifer. Fluorescence indicates the presence of glycoproteins. Fluorescent method. × 250.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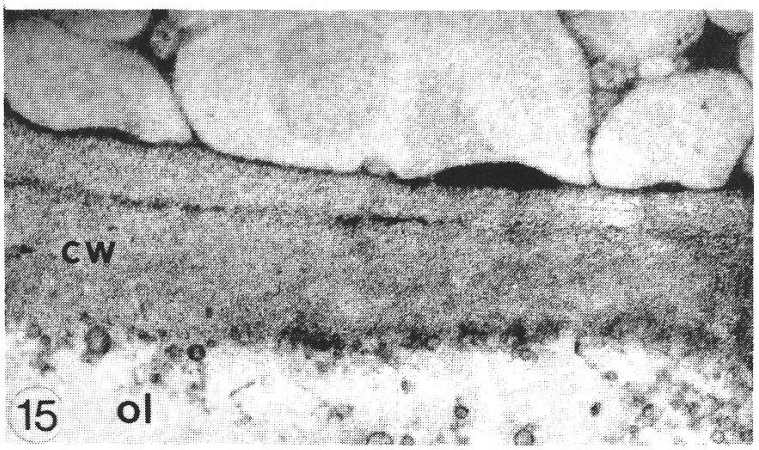

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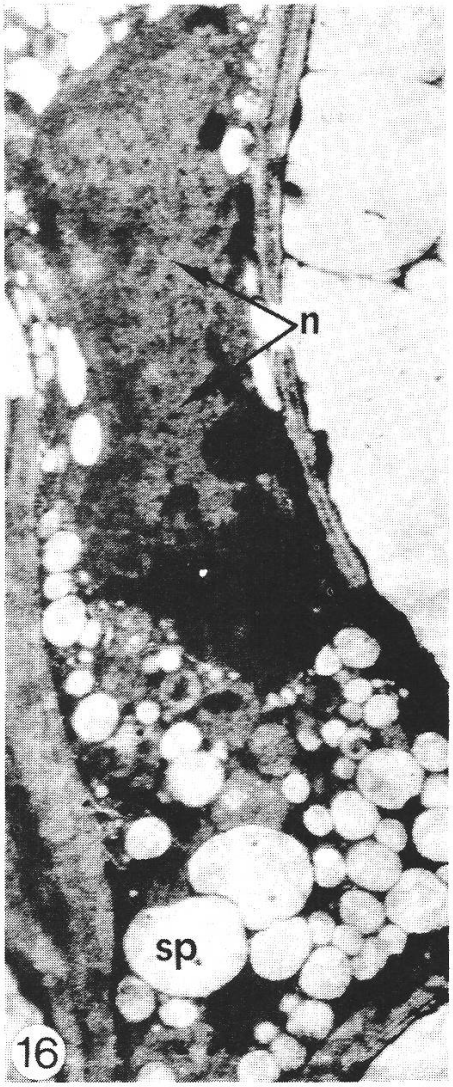

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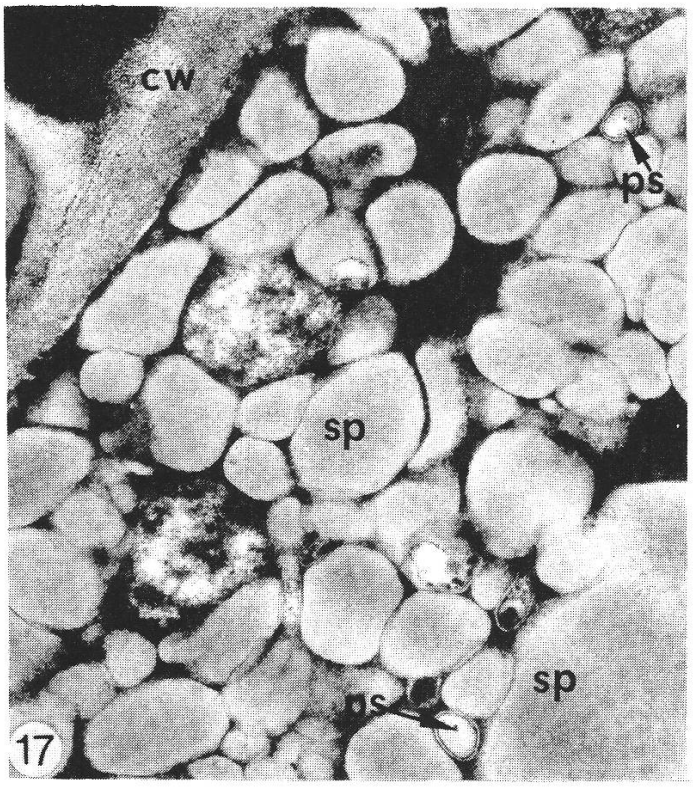

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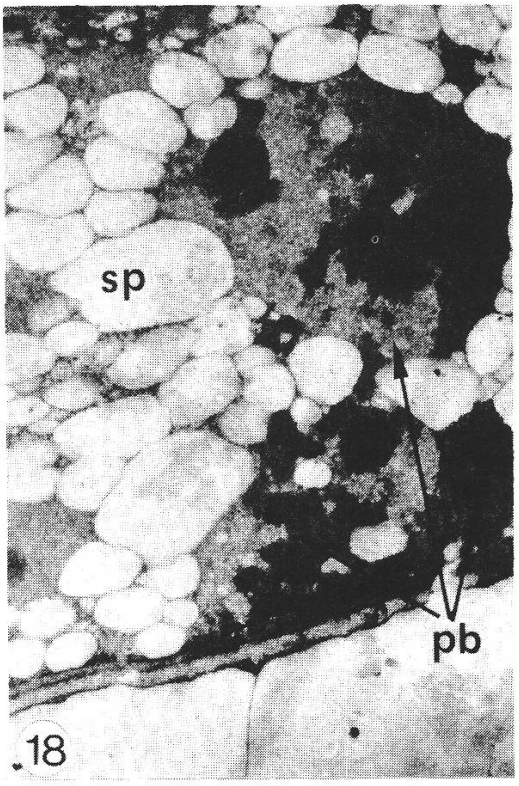

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Fig. 14. Electron micrograph showing transverse section of an innermost laticifer. Spherosomes in laticifer are small compared with those in adjacent cells. × 5,000.

Fig. 15. Transverse section of cotyledonary laticifer. Cell wall of outermost laticifer appears very thick. × 32,000.

Fig. 16. Longitudinal section of cotyledonary laticifer showing an elongated nucleus and several spherosomes of different sizes. × 9,500.

Fig. 17. Electron micrograph of an outermost laticifer. Note the presence of prospherosomes showing a thickened middle membrane layer. × 18,000.

Fig. 18. Transverse section of an innermost laticifer showing structure of protein inclusions and spherosomes of different sizes. × 12,000.

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prove that, in the cytoplasm of innermost laticifers, a large amount of sulphated proteins has accumulated (Fig. 8), while in outermost laticifers their concentration is very low. Fluorescence microscopy also permits the localization of a high content of glycoproteins in inner laticifers (Fig. 13). Silver impregnation, ninhydrin-Schiff's method and Hg-fluorescamine (Table I) indicate that no substantial difference exists between inner and outer laticifers with respect to the protein content of the cytoplasm. F-PAS reaction for insoluble carbohydrate shows a noticeable difference between inner and outer laticifers. The cytoplasm of the outermost laticifers exhibits a green fluorescence, while that of the inner ones appears orange-red in colour (Figs. 10, 11). Granules of starch were not observed in any part of the embryo.

With respect to the cell wall, electron microscopy discloses a clear difference in wall thickness between inner and outer laticifers. Observations carried out on a large number of sections have shown that the cell wall of the outermost laticifers (Fig. 15) is thicker than that of the innermost laticifers. The same difference also becomes visible by the F-PAS reaction (Fig. 12). The polychromatic properties of this method permit one to distinguish the inner laticifers, the walls of which are green, from the outer ones where the wall fluoresces orange-red.

Discussion

The absence of starch, phenol-carbohydrate complexes, polyterpenoids and lutoids in the laticifers of the mature embryo of *E. marginata* proves that, at this stage, these tissues are not yet completely differentiated. On the basis of histochemical and ultrastructural data, however, outermost and innermost laticifers appear to have a different functional role during embryogenesis. In particular, the innermost laticifers accumulate sulphated proteins, glycoproteins and carbohydrates in a complex form suggesting a role of storage significant for the formation of a definitive latex system during germination. It is well known (Hultin, 1972) that sulphated proteins are important in ribosome activation and that glycoproteins may be quickly dismantled and the derived products quickly utilized in various metabolic pathways (Brown and Kimmins, 1977). Therefore, the presence of storage compounds may furnish an explanation of the rapid activation and differentiation of innermost laticifers during germination. In fact, sulphated proteins and glycoproteins may be a transitional molecular stage suitable for furnishing a partially assembled material to the differentiating laticifer even in the absence of a well activated synthetic machinery. Outermost laticifers, in turn, do not show a particular cytoplasmic reactivity to the tests employed, but have a thick cell wall very sensitive to the F-PAS reaction. Under the electron microscope, the cell wall of embryonic laticifers appears to lack plasmodesmata; therefore, the exchange of compounds between laticifers and their adjacent cells remains an unsolved aspect of the problem of apparent «autonomy» of non-articulated laticifers during their subsequent growth. A possible answer to this question may be supplied by the thickness and the presence of a high number of 1,2 glycol groups in the cell walls of outermost laticifers. These characteristics are interpreted as indications of an active exchange among contiguous cells (Narthey, 1978). The role of outermost laticifers in the uptake of nutrients is indirectly supported by ontogenetical and topographical data and furnishes a coherent explanation of the temporary formation of a superficial network of these laticifers during embryogenesis (Mahlberg and Sabharwal, 1968; Bruni *et al.*, 1978).

By acridine orange, silver impregnation and electron microscopy, the nuclei of inner and outer laticifers appear different in number, shape, size and chromaticity. The strong metachromasy and the morphology of nuclei and nucleoli suggest a more active metabolism in outer laticifers. Support for this hypothesis is also furnished by the capacity of these nuclei to take up the dyes described for embryonic radicle cells (Villiers, 1972), shoot apical cells (Cottingnies, 1977), and in apple embryos (Dawidowicz-Grzegorzewska and Zarska-Maciejewska, 1979). This is considered an indication of a metabolically active state of cells.

In conclusion, it should be emphasized that outermost laticifers probably have a haustorial function in taking up embryonic and extraembryonic compounds during later embryogenesis and seed germination. Innermost laticifers on the other hand accumulate storage substances and appear destined for post-germination activity. The rapid activation of the latex system during germination of *E. marginata* (Mahlberg, 1959), therefore, seems to be a process well prepared even before germination.

Acknowledgements

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Résumé

Analyse histochimique et ultrastructurale des laticifères de Euphorbia marginata dans des embryons dormants.

Les aspects cytoloqiques et histochimiques des laticifères d' *Euphorbia marginata* ont été étudiés am moyen de la microscopie photonique, à fluorescence et électronique. Les résultats obtenus indiquent que les laticifères peuvent être distingués des cellules adjacentes par les petites dimensions des inclusions ergastoplasmiques et par le contenu élevé en protéines de la matrice cytoplasmatique. Des différences de morphologie et de contenu ont été observées entre les laticifères les plus intérieurs et les plus extérieurs. Les plus intérieurs contiennent des glycoprotéines et des protéines sulfurées; ils ont des noyaux avec des nucléoles «solides» et une paroi cellulaire mince. Les plus extérieurs montrent une paroi cellulaire plus épaisse, une cytoplasme fortement positif à la réaction PAS fluorescente et des noyaux avec des nucléoles vacuolisés. Ces différences sur le plan cytologique et le plan histochimique suggèrent, conformément aux données ontogénétiques obtenues précédemment, un rôle métabolique différent dans l'embryon entre les laticifères intérieurs et les laticifères extérieurs.

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