ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 50, nr 1—2: 21—28
1981

Polysaccharides and lipids in microsporocytes and tapetum of *Rhoeo discolor* Hance. Cytochemical study

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Abstract

The present report, which mainly presents cytochemical results, establishes the determination, localisation and evolution of the cytoplasmic and wall polysaccharides and lipids in the microsporocytes and the plasmodial tapetum of *Rhoeo discolor* Hance. Enzymatic controls, use of autoradiographic methods, and electron microscopy, have proved the validity of our cytochemical results and permitted to precise these results. In the microsporocytes and pollen grains, the callosic special wall, the carotenoid exine, the pectocellulosic intine, and the callosic and pectic young curved wall between the generative cell and the vegetative cell, have mainly held our attention. As for the tapetum which remains poor in insoluble polysacharides till the ultimate stages of microsporogenesis, it grows richer in choline — phospholipids during meiosis and, more lightly, in carotenoids beyond the tetrad stage; the periplasmodium does not seem to participate directly in the increase of exine lipids.

INTRODUCTION

Studies on pollen ontogenesis and on the relationship between tapetum and microsporocytes during the pollen ontogenesis have been approached in electron microscopy (EM) by several authors (secretory tapeta: Heslop-Harrison, Dickinson, 1969, *Lilium sp.;* Risueño et al., 1969, *Allium cepa;* Echlin, Godwin, 1968, 1969, *Helleborus foetidus;* Lombardo, Carraro, 1976, *Antirrhinum majus;* plasmodial tapeta: Mepham, Lane, 1968, 1969, 1970, *Tradescantia bracteata;* Roland-Heydacker, 1979, *Mahonia aquifolium*). In this field of pollen ontogenesis, the evolution of lipids and/or polysaccharides in cytoplasm and membranes of microsporocytes (with the associated study of the corresponding tapetum) has been the subject of some cytochemical investigations by light microscopy (see Remarks and Discussion) and some too rare autoradiographic studies (Southworth, 1971; Albertini, Souvré, 1978).

We have undertaken an elaborate study of "the pollen ontogenesis in relation with the tapetum" on *Rhoeo discolor* Hance with plasmodial tapetum. For the first time in this field we have associated cytochemical techniques in light microscopy (with enzymatic controls), histoautoradiography and electron microscopy (EM). The present note which mainly exposes the cytochemical results, discusses the determination, the localisation and the evolution of the polysaccharides and lipids in the cytoplasm of the tapetum and in the wall of the microsporocytes of anther of *Rhoeo discolor*. The use of histoautoradiographic studies (^{3}H-glucose, ^{3}H-myo-inositol, ^{3}H-acetate, ^{3}H-choline, ^{14}C-mevalonate) and of EM has allowed us to prove the validity of our cytochemical results and to precise these results.

MATERIAL AND METHODS

1. *Cytochemistry.* Sections (8 µm) of *Rheo discolor* Hance anthers, cut off at different stages, have been fixed with ethanol-acetic acid (3 : 1) for the polysaccharide and protein detection, or with calcium formol for lipid detection. These sections were stained by application of the cytochemical methods indicated in Table 1. Enzymatic controls (callase, cellulase, endo-polygalacturonase (endo-PG), pectine-methylesterase (PME), have often allowed for the improvement of the characterisation and of cytoplasmic wall compounds.

2. We have made an *autoradiographic* study, by variable time applications of different precursors of polysaccharides (D-(^{3}H-6) glucose: 25 µCi/ml; myo-(2-^{3}H)inositol, precursor of pectic compounds: 20 µCi/ml) and of lipids (^{3}H-sodium acetate: 20 µCi/ml; (^{3}H-methyl)-choline, precursor of choline-phospholipids: 20 µCi/ml; DL-(2-^{14}C)-mevalonic acid, DBED salt, precursor of sterols and carotenoids: 2 µCi/ml). Some excised anthers have been brought into contact with the precursors (A l b e r t i n i, 1971). Sections (8 µm) have been subject to the autoradiography method of F i c q (1961) (Ilford K2 emulsion in gel form for the tritiated compounds, exposure time: 4 weeks; Ilford G5 emulsion in gel form for ^{14}C-mevalonate, exposure time: 2 weeks).

3. Observations have been made by *EM* (paraformaldehyde-osmium fixation: Epon embedding). Some sections were treated with periodic acid-thiocarbhydrazide-silvers proteinate (PATAg) for polysaccharides detection (T h i é r y, 1967).

RESULTS

The results of cytochemical studies are given in Table 1 (meiocyte, microspore and pollen walls), and in Table 2 (tapetum cytoplasm).

Table 1

Chemical characterisation of the walls of meiocytes, microspores and young pollen grains of *Rhoeo discolor* by the application of cytochemical methods

Stage and wall / Stain	Premeiotic resting stage	Synizesis	Diakinesis -anaphase 1		Diad stage	Tetrad stage	Micro-spores (stages 1-2)	Micro-spores (stage 3)		Microspores (stages 4-6)		Young pollen		
	pp	pp	pp	ps	ps and pl 1	ps, pl 1 and pl2	ex	ex	in	ex	in	ex	in	plp
P.A.S. (P.A.S.+polysaccharides are red) (Mac Manus, 1948)	++	++	++	++	++	++	—	—	+	—	++	—	++	+
P.A.S. after callase (a)	++	++	++	—	—	—	—	—	+	—	++	—	++	—
P.A.S. after cellulase (b)	+	+	+	++	++	++	—	—	±	—	+[6]	—	+	±
P.A.S. after endo PG (c)	+	+	+	++	++	++	—	—	±	—	±	—	±	±
P.A.S. after PME (d)	++	++	++	++	++	++	—	—	+	—	++	—	++	+
Aniline blue (secondary fluorescence of callose is yiellow) (Currier, 1957)	—	—	—	+	++	++	—	—	—	—	—	—	—	++
Aniline blue after callase (a)	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ruthenium red (pectic compounds) (Johansen, 1940)	++	+	±	—	—	—	—	—	+	—	++	—	++	±
Alcian blue pH 2.5 (acid polysaccharides with COOH groups) (Wagner, Shapiro, 1957)	++	++	+	—	(pl 1: ++)[1]	(pl 1, 2: ++)[2]	—	—	+	—	++	—	++	+
Alcian blue after endo PG (c)	+	+	—	—	pl 1: —	pl 1, 2: —	—	—	—	—	—	—	—	±
Alcian blue after PME (d)	+++	+++	++	—	pl 1: +++	pl 1, 2: +++	—	—	++	—	+++	—	+++	+
Sudan black B (neutral lipids) (Baker, 1947)	—	—	—	—	(pl 1: +)[3]	(pl 1, 2: +)[4]	+	++	—	+++	—	+++	—	—
Nile blue (acid lipids) (Cain, 1947)	—	—	—	—	—	—	+	+	—	++	—	++	—	—
D.D.D. (proteins with free SH) (Barrnett, Seligman, 1952)	+	+	—	—	(pl 1: +)[5]	(pl 2: +)[5]	—	—	—	—	—	—	—	—
D.D.D. before cysteine (e)	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fast green FCF pH 8.0 8.1 (basic proteins) (Alfert, Geschwind, 1953)	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Alloxane Schiff (total proteins) (Yasuma, Ichikawa, 1953)	—	—	—	—	—	—	—	—	—	—	—	—	—	—

pp — primary wall; ps — special perimeiocyte wall; pl 1 — cell plate 1; pl 2 — cell plates 2; ex — exine; in — intine; plp — pollen cell plate;
a — callase (1 mg/ml), pH 7.5, 38°C, during 2 hours;
b — cellulase (10 mg/ml), pH 4.7, 25°C, during 21 hours;
c — endo-PG (endo-polygalacturonase) (10 mg/ml), pH 4.6, 25°C, during 16 hours;
d — PME (pectin-methylesterase) (0.02 mg/ml), pH 7.9, 30°C, during 2 hours;
e — cysteine (1%, pH 8, 50°C, 3 hours) applied after the D.D.D. action and before staining with Diazo blue B (inhibition by competition).
+++ — high level of stain; ++ — clear stain but with a medium intensity; + — low stainability; ± — little or not stianed; "—.. — no stain; 1 — the cell plate of the diad (essentially the middle part) is stained by Alcian blue; 2 — the cell plates of the tetrad only are stained (essentially the middle part) by Alcian blue; 3 — Sudan black B stains only the middle part of cell plate; 4 — Sudan black B stains only the middle parts of the cell plates; 5 — young cell plate 1 or 2 is positive; 6 — the inner layer of the intine is P.A.S — negative after action of cellulase, the outer layer remains positive.

Table 2
Cytochemical study (polysaccharides, lipids, proteins) of the tapetal cytoplasm of *Rhoeo discolor*

Stain \ Stage	Premeiotic resting stage	Synizesis	Diakinesis -anaphase 1	Diad stage	Tetrad stage	Micro-spores (stages 1-2)	Micro-spores (stage 3)	Micro-spores (stages 4-6)	Young-pollen Cyt. (3)	Young-pollen Gran. (4)
P.A.S. (1)	±	±	±	±	±	±	±	±	±	++
P.A.S. after callase	±	±	±	±	±	±	±	±	±	++
P.A.S. after cellulase	±	±	±	±	±	±	±	±	±	++
P.A.S. after endo-Pg	±	±	±	±	±	±	±	±	±	±
P.A.S. after PME	±	±	±	±	±	±	±	±	±	++
Aniline blue (1)	−	−	−	−	−	−	−	−	−	−
Ruthenium red (1)	±	±	±	+	+	±	−	−	−	−
Alcian blue (1)	−	−	−	−	−	−	−	−	−	−
Alcian blue after endo-PG	−	−	−	−	−	−	−	−	−	−
Alcian blue after PME	−	−	−	±	±	±	±	±	±	−
Sudan black B (1)	++ (2)	++ (2)	++	++	++	+	±	±	±	−
Nile blue (1)	+ (2)	+ (2)	++	++	++	++	+	+	+	−
D.D.D. (1)	+ (2)	+ (2)	+ (2)	+ (2)	++	++	+	+	±	+
D.D.D. before cysteine	±	±	±	±	±	±	±	±	−	−
Fast green FCF (1) (Alfert, Geschwind, 1953)	+ (2)	+ (2)	+	+	++ (2)	+	±	±	±	+
Alloxane-Schiff reaction (1) (Jasuma, Ichikawa, 1953)	++	++	+	++	++	+	±	±	±	±

(1) — the references for the Material and Methods, the level of stainability (−, ±, +, ++) and the conditions of the enzyme applications are indicated in Table 1;
(2) — stainability of the tapetal cytoplasm is higher than that of the pollen mother cells;
(3) — cytoplasmic coloration other than that of granules;
(4) — cytoplasmic granules.

Fig. 1. Tetraspore with primexine (arrow)
The callosique special plates 1 and 2 (pl 1 and pl 2) and the starch of the amyloplasts (a) react positively to the PATAg test. (N): nucleus. Fixation: paraformaldehyde-osmium (× 12500)

Fig. 2. Periplasmodium during tetrad stage
The dimensions and the density of the organelles show a great biosynthetic acti-vity. Dictyosomes (d), proplastide (p), mitochondria (m), rough endoplasmic reticulum (RER), polyribosomes. Fixation: paraformaldehyde-osmium; uranyl acetate-lead citrate (× 40000)

The walls of meiocytes, microspores and pollen grains

The special wall (more particularly the external dense layer of the meiocyte envelope beyond the pachytene, and the cell plate (s)), stainable by PAS and producing a yellow fluorescence with aniline blue is essentially of callosic nature (Fig. 3), its stainability by the two reagents becoming null after the application of callase in enzymatic control (Table 1).

The microspore and pollen exine only well stainable by Nile blue and Sudan black B and, during its formation, intensively incorporating ^{3}H-acetate (Fig. 7) and ^{14}C-mevalonate (personal observations not yet published), is essentially of lipid and more specially corotenoid nature.

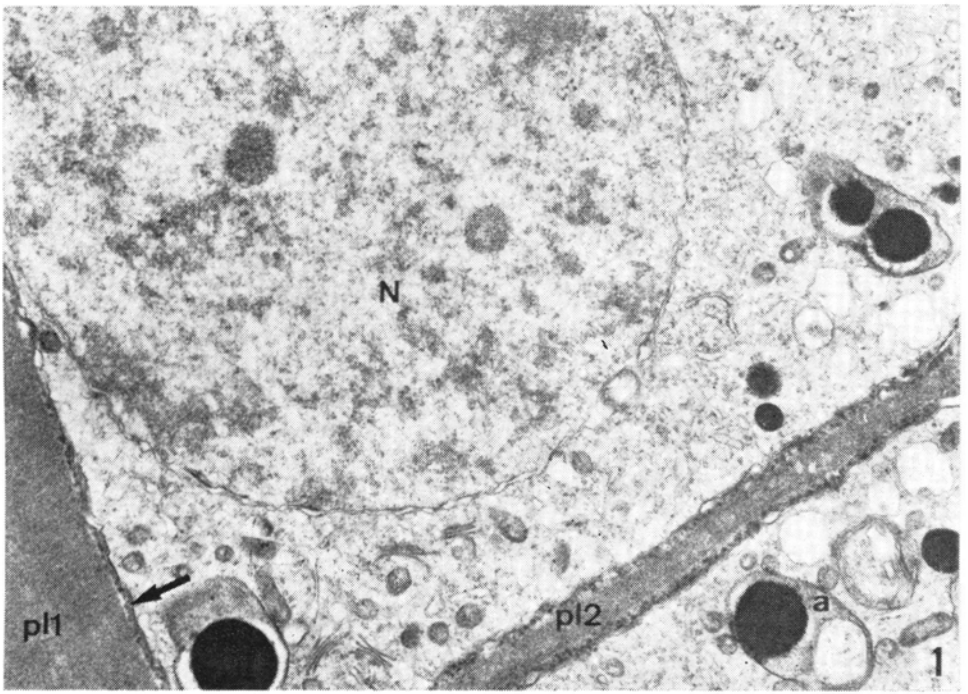
The intine which contains no callose at any stage of its evolution is pecto-cellulosic (Table 1). The outer layer of intine is principally pectin, as its stainability by PAS is not weakened by cellulase but is well reduced by endo-PG. As for its inner layer, it is made up of pectic compounds (more particularly with free carboxylic groups) and of cellulose.

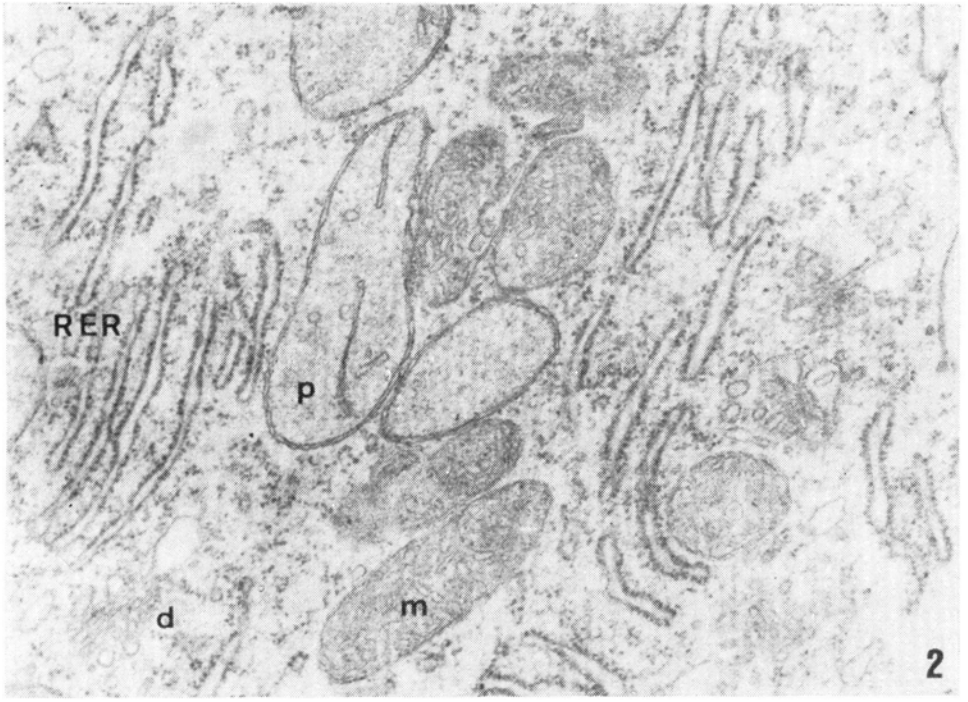
The wall between generative and vegetative cells of pollen grain is, in its young phase, a fine curved cell plate supported by the intine and enclosing the generative cell: cellulose and a big amount of pectic compounds (more particularly with free carboxylic groups) and callose then go into its constitution (Fig. 4, Table 1).

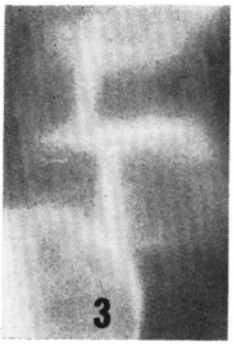
Tapetum cytoplasm

During microsporogenesis, the cytoplasm of plasmodial tapetum of *Rhoeo discolor* remains poor in insoluble polysaccharides. Indeed, during this entire period, it only slightly incorporates the ^{3}H-glucose (Fig. 5 and Albertini, Souvré, 1978), and is not or poorly stained by PAS, Lugol and the other stains used for carbohydrates (Table 2 and Albertini, Souvré, 1978). On the other hand, the tapetal cytoplasm is quite rich in acid and neutral lipids during the whole meiosis

PLATE I



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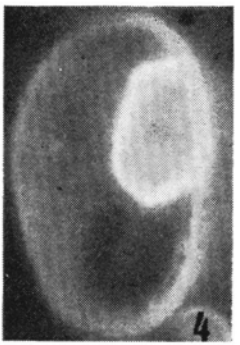
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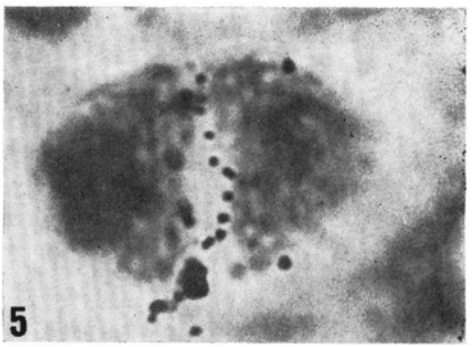
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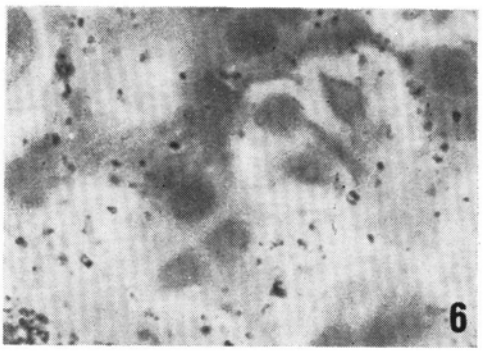
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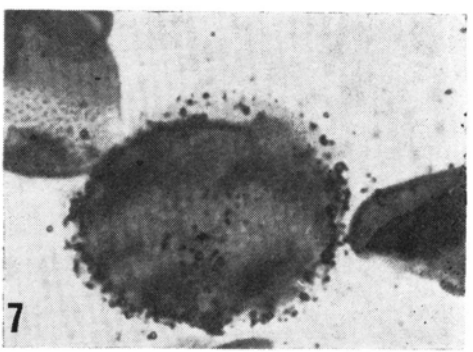
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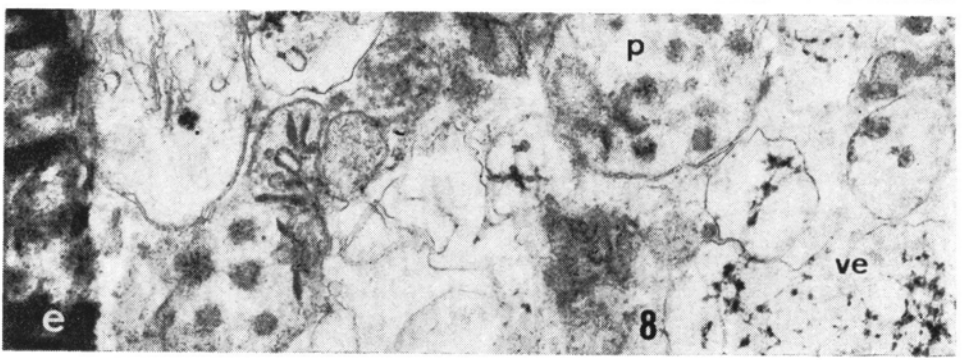
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Fig. 3 and 4. The secondary fluorescence of callose with aniline bue. Fixation in ethanol:acetic acid (3 : 1) (× 2200)

Fig. 3. Fluorescent special wall of the tetrads. Fig. 4. The young curved wall between generative and vegetative cells of the young pollen presents a clear fluorescence.

Fig. 5. ^{3}H-glucose: at the beginning of division II, the meiocyte cell plate 1 is (20 µCi/ml) during 3 h on excised anthers. Stain with methyl green-pyronin

Fig. 5. ^{3}H-glucose: at the beginning of division II, the meiocyte cell plate 1 is well labelled. Fixation in ethanol:acetic acid (3 : 1) (× 3600). Fig. 6. The plasmodial tapetum incorporates ^{3}H-acetate actively; notice the tetrad inactivity (× 1800).

Fig. 7. The old fertile microspore incorporates ^{3}H-acetate in its exine when the sterile microspores are inactive. Fixation in calcic formal (× 1800).

Fig. 8 and 9. Old microspore

The tapetum degenerates; it is very vacuolised and shows plastids (p) containing polysaccharides (PATAg-positive test in Fig. 9), vesicules (ve) the granulous and fibrous content of which is osmiophile, and lipid globules (l). (e): exine. Fixation: paraformaldehyde-osmium; Fig. 8: uranyl acetate-lead citrate (× 20000)

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and the two first stages of microspores as shown in Table 2, and by the strong incorporations of ^{3}H-acetate (labelling all the lipids) and of ^{3}H--choline (labelling the choline-phospholipids) (personal observations not yet published).

At the stage of young pollen grain, this plasmodial cytoplasm becomes rich in "granules" stainable by PATAg, by PAS (polysaccharide granules of which the clear stainability by PAS is strongly reduced after action of endo-PG: Table 2) and by the protein stains (DDD and Fast green FCF, pH — 8), not stained by the lipid reagents: therefore these granules seem essentially of glycoprotein nature.

REMARKS AND DISCUSSION

The walls of meiocytes, microspores and pollen grains

We have verified the results obtained previously (Albertini, Souvré, 1978) on *Rhoeo discolor*: the deposits of perimeiocyte callose and meiotic cell plates 1 and 2, fluorescent in aniline blue, are PAS positive. Such a result, which is far from the conclusion (callose PAS--negative) of Heslop-Harrison (1964), is proved by the fact that this meiocyte callose is also PATAg-positive (Fig. 1). This agrees with the results obtained on other monocotyledones (*Allium cepa*: Nanda, Gupta, 1974; *Zea mays*: Panchaksharappa, Rudramuniyappa, 1974). The essentially callosic middle part of the meiotic cell plates 1 and 2 contains, above all during its initial phase, acid polysaccharides with carboxylic groups (little or not esterfield), proteins with -SH, and lipids (Table 1). Similarly as in *Tradescantia reflexa* (Idelman,

1958), the existence, in the middle part, of a lipoprotein-SH complex, perhaps associated with pectic acids, is not to be put aside.

In its outer layer the PAS-positive intine, without callose is rich in pectic compounds which have free carboxylic groups and in pecto-cellulose in its inner layer, the latter increasing during the phase of "microspore (st 4/5)-young pollen". Contrary to the opinion of Waterkeyn (1964), for whom the intine is essentially callosic, our analysis agrees with those of Sitte (1953), of Mepham and Lane (1970) and of Southworth (1973).

The wall between generative and vegetative cells of the pollen grain would be essentially callosic during its young phase (Mepham, Lane, 1970, *Tradescantia bracteata*), pecto-cellulosic (Maruyama, 1966, *Tradescantia paludosa*). In *Rhoeo discolor*, it seems to be undoubtedly pecto-callosic. This fine wall progressively looses its pecto-callosic constitutive elements during the pollen grain maturation and comes off the intine in order to surround the generative cell completely. The generative cell and the vegetative cell are then separated only by their plasmalemma (personal observations by EM not yet published).

Role and activity of the plasmodial tapetum

During the whole microsporogenesis, the cytoplasm of plasmodial tapetum, through which the pool of carbohydrate precursors is transfered to the microsporocytes, is clearly less rich in insoluble polysaccharides (the lack of amyloplasts should be underlined, Fig. 2) than the amyloplasts and the walls of the microsporocytes (Fig. 5, Table 1 and 2; Albertini, Souvré, 1978). From the meiotic prophase to the last microsporal stage, the periplasmodium only seems to retrocede simple soluble carbohydrates to the meiocytes and microspores. The carbohydrate polymers in the walls and in the plastids of the microsporocytes have essentially an endogenous origin. Later (last microsporal phase), the tapetum gets richer in polysaccharides (stainable by PATAg (Fig. 8 and 9) and by PAS) included in vesicules of plastid type (with double membrane, Fig. 9), which soon evolve in glycoprotein spherical granules (Table 2), of 1 µm of diameter, visible around the young pollen grains. These granules which constitute the essential part of the tapetal tryphine of *Rhoeo discolor* adhere to the pollen exine before and during anthesis.

In the cytoplasm of periplasmodium, the amount of acid and basic proteins increases during meiosis to reach a maximum during the peroid "tetrad stage — stage 2 of microspores" (Albertini, 1970 and Table 2). The evolution of the amount of acid and neutral lipids in the cytoplasm of tapetum is close to the precedent one, as shown in Table

2 and by the incorporations of ^{3}H-acetate (Fig. 6) and of ^{3}H-choline which are high during meiosis and at the tetrad stage. These cytochemical and autoradiographic results are in agreement with our results in EM. In fact, during meiosis, we observed a significant development of the cytomembranes in the tapetum which were essentially of protein and phospholipid nature. These cytomembranes belong mainly to the rough endoplasmic reticulum, to the mitochondria, and to the proplastids (Fig. 2).

The number of lipid globules which is insignificant during meiosis, increases from the tetrad stage in the tapetal cytoplasm, it would seem, at the moment when this cytoplasm begins to incorporate ^{14}C-mevalonate, precursor of carotenoids, significantly (personal observations not published). These globules (per unit of cytoplasmic volume) are less numerous in the tapetum than in the vegetative cell of young pollen.

The fertile microspores and the young pollen grain incorporate, during their development, the ^{3}H-acetate (Fig. 7) and the ^{14}C-mevalonate at the exine level. Basing on the results of the cytochemical tests for lipids (progressive increase of Sudan black B stain during microspore and pollen evolution; see Table 1) we may assume that exine would probably get richer in carotenoid sporopollenin after elimination of the special callosic wall.

Two facts show that the progressive lipid increase in the exine after the tetrad stage has its origin directly in the gametophyte: the density of lipid globules is higher in the cytoplasm of the vegetative cell of young pollen than in the tapetal cytoplasm and, above all, the labelling of the exine by ^{3}H-acetate (Fig. 7) and ^{14}C-mevalonate observed exclusively on the fertile microspores and pollen grains which, unlike sterile microspores, keep a functional genome and cytoplasm. The plasmodial tapetum of *Rhoeo discolor* does not seem to be the builder of the exine at any time: it would merely provide simple lipid molecules to the gametophyte which would be used by the latter for the elaboration of the exine. Our results agree with the authors working on plasmodial tapeta (Mepham, Lane, 1968, 1969, *Tradescantia bracteata;* Roland-Heydacker, 1979, *Mahonia aquifolium*).

Acknowledgements

This research was supported by the C.N.R.S., France (RCP no 574).

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